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Flavor and Health Benefits of Small Fruits

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Flavor and Health Benefits of Small Fruits

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Foreword

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Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

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Preface

Epidemiological and scientific studies have provided data in support of current drive to include fruits in daily diets, for health benefits. Small fruits, particularly small berries, have attracted much attention in the recent years and have been associated with reduced risk of cardiovascular diseases, cancer, and other chronic disorders. The increasing appeal of small fruits has prompted research to improve flavor quality, which is largely due to the volatile constituents in the fruits. In general, variability and content of volatile constituents determine fruit flavor while non-volatile constituents determine the health effects of fruits. Some volatile constituents have health promoting properties. Similarly, some non-volatile compounds also contribute to fruit flavor. These diverse topics are covered succinctly in this book.

This book is unique, having compiled research works on flavor together with health benefits of small fruits in one volume. The chapters were written by speakers who were invited and presented some of their latest and greatest research results in the Agricultural and Food Chemistry Division symposium “Flavor and Health Benefits of Small Fruits” at the 236th American Chemical Society National Meeting and Exposition held in Philadelphia, Pennsylvania on August 17-21, 2008.

One section of this book is focused on volatile constituents, and includes chapters on identification and characterization of volatiles for the improvement of flavor as well as aroma of the fruits. Chapters describing the health benefits of small fruits, and specific constituents, on dislipidemia, tumor, and microbial disease are put together in another section. Some clinical studies are also reported. The last section of this book includes chapters discussing new biological testing and analytical methods, and engineering of biosynthetic pathways to increase the production of bioactive and volatile constituents. This work exemplifies what could become the means to enhance fruit flavor quality and health-beneficial phytochemicals in the future.

We are grateful to the authors for their contributions as well as the reviewers for their valuable critiques.

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Chapter 1

Flavor of Small Fruits

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This review focuses on presenting an overview of the recent research published on the flavor of small fruits, primarily strawberry, raspberry and blackberries. The work that has been published primarily involves studies either on the linkages between genetics and flavor formation (strawberry), or efforts to characterize the flavor compounds differing between well liked varieties of berries and more robust varieties. Very little information has been published on other small fruits.

There is surprising little research published on the flavor chemistry of small fruits. The last major symposium in this area was hosted by the American Chemical Society in 1993 (*1*). The proceedings of this symposium covered analysis, adulteration, biogenesis and biotechnology, precursors and environmental, maturity and varietal effects on fruit flavor. Of the 25 chapters in this book, only three considered small fruits, i.e. berries and grapes, the topic of this paper. Since this symposium, some work has been published on the chemistry of small fruit flavor but the number of papers in print is small (Figure 1) averaging only about 4 papers per year (SciFinder Scholar, American Chemical Society, 2007).

The early research on the chemistry of fruit flavor focused on identifying the volatiles that characterize the product. Currently most of the key volatiles characterizing the primary fruits (commercial importance) have been identified.

Therefore, research on fruit flavor now generally focuses on a few of the lesser known fruits or determing metabolic pathways and genetic liknkages for flavor formation. While the greatest effort has been devoted to strawberries and then grapes, other berries have been receiving some attention recently. This review will summarize some of the recent work (last 10 years) done on the flavor chemistry of small fruits.

Analytical Methods

The analytical methods applied in fruit flavor research have followed a similar trend as flavor research on other foods progressing from distillation and solvent extraction methods to the very commonly used absorption methods (*2*): SPME is the most commonly used methodology for flavor isolation. Every author justifies this choice in the same manner, e.g. the method is solvent-less (implications in terms of environment and no solvent peak to hide the early flavor profile), simple, readily automated and is relatively inexpensive (*3*). There have been numerous papers devoted to optimizing this method for a given flavor analysis. Interestingly, the papers have optimized the method based on GC profile as opposed to sensory profile. One of the basic considerations in evaluating an aroma isolation method is to determine by sensory evaluation if the aroma isolate prepared is reminiscent of the food (or off flavor in the food) being studied. Rega et al, (*4*) have addressed this oversight in their work on orange juice where they optimized the method based on sensory properties of the recovered volatiles.

Despite the very broad use of SPME in flavor analysis, the method has numerous weaknesses that seem to be ignored by most researchers. Negatives of this method include competitive binding of volatiles, deterioration on use (may be used for 100 injections) may result in changing performance or fiber breakage, and very limited phase volume which limits the technique to only the more abundant, non-polar volatiles (*5*, *6*). Also, if the fiber is placed in a fat-containing sample, the lipids will also be absorbed and create artifacts during thermal desorption.

These limitations have lead to the use of absorption methods that have larger phase volumes, e.g. Solid Phase Extraction (SPE) or Stir Bar Sorptive Extraction (SBSE), which then result in better recoveries and less discrimination. Some of the papers to be discussed later in this paper (notably those from Qian's group) have used SPE and SBSE to study the flavor of some berries.

In terms of instrumentation used in flavor studies, the standard today is still normal GC-MS. We are seeing some Time of Flight MS instruments and multidimensional GC in use (*7*). These instruments permit gathering more reliable data.

Strawberry Flavor

As noted earlier, of the small fruits/berries, strawberries have received the most attention. Much of the initial work on strawberries focused on identifying the volatiles that characterize the product. More than 360 volatiles have been identified in strawberries (*8*, *9*). Of these volatiles, Schieberle and Hofmann (*10*) have proposed that 15 are the primary contributors. This group consisted of esters (ethyl and methyl butanoate, ethyl and methyl-2-methylbutanoate,

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ethyl and methyl-3-methylbutanoate, and ethyl-2-methyl propanoate (all have fruity notes), an aldehyde (*Z*-3-hexenal (green)), acids (acetic, butanoic, 2- and 3-methylbutanoic acids (sweaty)), a diketone (2,3-butanedione (buttery)), and two furanones (4-hydroxy-2,5-dimethyl-3(2H)-furanone and 2,5-dimethyl-4-methoxy-3(2H)-furanone (caramel-like)). While there is likely still a need to do further work on identifiying and quantifying the volatles that characterize strawberries, this area of research has largely been inactive in recent years.

Current research on strawberry flavor is focused on establishing genetic links to flavor, or external factors that alter flavor development while on the plant. These efforts at times appear to be simply for academic interest (we do not understand how flavor is formed) and other times is aimed at improving the strawberry flavor. Similar to many other fresh fruits/berries, strawberries are typically bred for yield, disease resistance and stability in storage and transport. Wild strawberries may have a shelf life of one to two days while the cultivated species are expected to retain a desirable appearance for 1 week or more. The cost for this extended shelf-life has been immense in terms of texture and flavor quality. Today's strawerries are large and red: they are beautiful but the texture resembles that of an apple (it takes a good knife to cut it) and the flavor bears no resemblance to a traditional species. Unfortunately, flavor is not a high priority for growers.

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Flavor Formation and Genetics

As noted earlier, esters, carbonyls, acids, and furanones are considered key volatiles characterizing strawberry flavor(*11*, *12*) . Thus, it is logical that most of the genetic studies have focused on determining the genes responsible for the formation of these classes of compounds. One of the earliest studies found the genes responsible for the formation the enzyme SAAT (strawberry alcohol acyl-CoA transferase) considered largely responsible for the formation of esters in strawberry (*13*). Later, Aharoni et al. (*14*) identified strawberry genes associated with the production of enzymes leading to various terpenes. (While this group of compounds was not identified as being key contributors to strawberry flavor, many researchers feel they make a significant contribution to flavor) This enzyme (FaNES1 - Fragaria 3 ananassa nerolidol synthase) is capable of generating either linalool or nerolidol when given geranyl diphosphate (GPP) or farnesyl diphosphate (FPP), respectively, as substrate.

The greatest amount of effort in this area has been devoted to determining the genes and resultant pathways responsible for the formation of the furanones (e.g. (*15–18*). An enzyme (FaOMT - Fragaria 3 ananassa O-methyltransferase) that encodes an O-methyltransferase partially responsible for the biosynthesis of 2,5-dimethyl-4-methoxy-3(2H)-furanone was found by Lavid et al., (*19*) and Wein et al., (*20*). Very recently, Raab et al. (*15*) have reported identifying the Fragaria 3 ananassa quinone oxidoreductase gene (FaQR). This enzyme is proposed to catalyze the final step in the biosynthetic pathway leading to 4-hydroxy-2,5-dimethyl-3(2H)-furanone. Progress is being made in determing the genes responsible for the major contributors to strawberry flavor. The task remaines as to how to use this information to create a better flavored strawberry.

External Factors Influencing Strawberry Flavor

The influence of growing temperature, lighting, and gas environment on flavor formation (most commonly volatiles) have been reported. Sanz et al. (*21*) grew strawberries under different day/night temperature regimes (25°C/25°C, 25°C/15°C, 25°C/10°C, and 25°C/5°C) and monitored the formation of selected volatiles and non-volatiles (sugars, acids, and anthocyanins). Extreme day/night temperatures, 25°C/25°C and 25°C/5°C, had a substantial negative effect on aroma formation. 25°C/15°C and 25°C/10°C day/night temperatures produced a normal pattern of aroma formation with an increase in volatile content as fruit ripened. Maximum aroma production was observed when growing the berries at 25°C/10°C day/night temperatures.

Perez and Sanz (*22*) have reported on the use of high oxygen modified atmosphere storage on the shelf-life and quality of strawberries. They noted that the common practice of storing strawberries under low temperature (0-2C) and high CO_2 (15-20%) results in firmer fruit with less decay, however, the aroma becomes unacceptable. The berries tend to accumulate acetaldehyde, ethanol, and ethyl acetate formed through anaerobic respiration pathways (*23*). Based on related research on high oxygen storage, the authors felt that a high oxygen/high carbon dioxide combination environment may be more suitable for strawberry. Unfortunatley, these environments also resulted in the formation of off flavors, in fact, seeming to have an additive effect being worse than either environment alone.

The influence of lighting (more accurately, shading) during growing on strawberry flavor was studied by Watson et al. (*24*). This sudy is interesting in that the authors used a rapid method (Atmospheric Pressure Ionization Mass Spectrometry) to obtain a large amount of data on volatiles (13 key volatiles) for statistical treatment. The rationale for this work was to provide some understanding as to why the flavor of strawberries varies so greatly (investigated harvest date) and to offer some insight into the effects of growth environment (shading) on berry flavor quality. There is relevance to commercial practice in that berries may be grown in polyethylene tunnels which reduces lighting by 10% or in glass greenhouses which could reduce lighting by 30% or more (*25*). The shading treatment was 0, 25 or 47% shading for 2 weeks beginning 1 week prior to the first ripening. The harvest date study used berries that came to ripeness over a 12 day period.

The authors found that harvest date had a highly significant effect on both volatile and non-volatile compounds although there was no trend in the data other than sucrose level tending to decrease over the harvest period. Shading had a significant effect on hexanal, hexenal, ethyl methyl butyrate, and methyl butyrate concentrations at some harvests. In general, the higher the level of shading, the lower the level of the volatiles and non-volatiles in the fruit.

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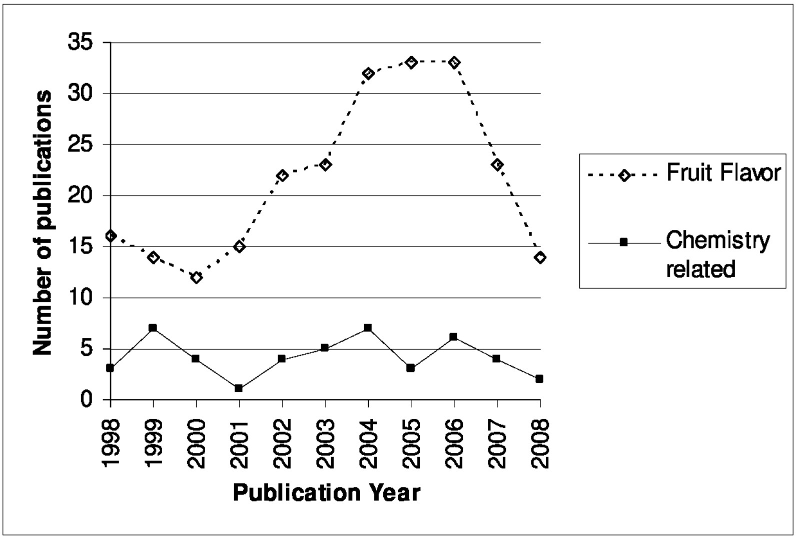

Figure 1. Numer of publications on the flavor of small fruits published annually (SciFinder Scholar, American Chemical Society, 2007)

Blackberry Flavor

Blackberries have received some attention over the years. The earliest work on blackberry flavor chemistry was done in Oregon State by some of the pioneers of flavor chemistry (*26–30*). As of the mid 90s, 147 volatiles had been identified but little work had been done to determine the characterizing compounds (*31*). Both Turemis et al. (*32*) and Qian's group (*33–35*) have focused on identifying the characterizing compounds in blackberry. Turemis et al (*32*) reported 5-hydroxymethyl furfural as the primary blackberry-like aromatic compound while Qian et al. have reported more broadly on odor active comounds. The rationale for Qian's work was that the flavor of the thorned Marion cultivar blackberry (Rubus *spp. hyb)* is preferred by consumers over the cv. Thornless Evergreen (Rubus *laciniatus Willd.*). Thorned berries are problematic in cultivation and harvesting and thus, research was initiated to relate blackberry flavor characteristics to berry genetic makeup ultimately breeding new thornless blackberry cultivars with Marion flavor.

Klesk and Qian (*33*) used a dynamic headspace method (Tenax trapping) in aroma isolation followed by OSME for determining potential importance in characterizing blackberry flavor. They added 22 previously unidentified compounds to the overall list of volatiles identified in blackberries and provided some insight into differences between cultivars. They noted that no single compound was found to be characteristic of blackberry aroma. In a subsequent study using a more rigorous method for aroma isolation (solvent-assisted flavor extraction) (*34*), they added another 27 compounds to the list of blackberry

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volatiles and provided more detailed insight into cultivar differences. With one exception, the two cultivars contained the same characterizing volatiles but their proportions were different. Their most recent publication on this berry (*35*) is a further study providing more detail on determing key volatiles that differ between these two cultivars.

Qian's group (*36*) has also published some work on another blackberry cultivar, Chickasaw blackberry (Rubus L.). This cultivar is the result of breeding programs at the University of Arkansas aimed at developing a berry more suited to commercial industry. Apparently this cultivar ripens very early and has attractive, large, firm fruit, with good flavor. Their work on this cultivar focused on comparing the key odorants in this cultivar when grown in Oregon Vs. Arkansas. They noted that similar volatiles were found with only subtle differences in quantitative data.

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Raspberry Flavor

Raspberry flavor chemistry has been extensively studied for many years. Issues of volatile identification (over 235 identified), maturity and numerous environmental factors have been studied by various researchers (*37*). The interest in new cultivars (quality or yield), selection of disease resistant cultivars, effect of growing location, or efforts in flavor-related gene identification and breeding programs has resulted in continued study.

Klesk et al. (*37*) used SAFE extraction followed by GC-MS and GCO (AEDA) methods to compare the flavor profiles of Meeker raspberries grown in Oregon and Washington. The volatiles found were similar across growing locations with some differences in the amounts of each volatile found. The results were quite consistent with previous research.

In a recent study, Malowicki et al. (*38*) used SBSE for volatile isolation and GC-MS for the identification and quantification of volatiles in Chilliwack, Tulameen, Willamette, Yellow Meeker, and Meeker raspberry cultivars. The method yielded quantification limits of 1 μg/kg for most compounds, however, raspberry ketone and zingerone were poorly recovered. Quantitative data showed that volatile concentrations varied across cultivars with particularly large variations in β-ionone, α-ionone, geraniol, linalool, and (*Z*)-3-hexenol concentrations. Their observation that both growing location and cultivar affect the isomeric ratio of linalool was of interest.

Changes in cultivars and increased planting densities tend to favor the spred of diseases. The rapid increase of raspberry bushy dwarf virus (RBDV) is an example. This virus occurs naturally worldwide and can infect most bramble species and cultivars. RBDV causes dwarfing and shoot proliferation, reduces cane vigor, and causes leaf discoloration. Decreased drupelet set results in smaller berries that do not hold together and these berries can only be sold for processing as juice, puree,or jam, which reduces the value of the crop. The primary means of managing this virus is through planting disease resistant cultivars.

Genetic modifications were made to ‘Meeker’ red raspberries to impart RBDV resistance. In order to determine the effect of this genetic modification on berry flavor, the RBDV-resistant transgenic and wild type ‘Meeker’ plants were grown in Oregon and Washington and then studied for flavor profile (*39*). SBSE extraction was used for volatile isolation and gas chromatography-mass spectrometry (GC-MS) was used in identification and quantification. The authors found very few differences in volatile concentrations between the transgenic varieties and the wild type ‘Meeker’: Much larger variations were found between growing sites and harvest seasons. The Oregon raspberries had slightly higher sugar levels and lower titratable acidities than the Washington raspberries.

Flavor of Other Small Fruit

There is additional information in the literature on a number of other small fruit. Very often these fruit are analyzed more as a curiosity than for any other objective. There are numerous publications on grapes but primarily due to their use in making wine. Surprisingly, there are few publications on blueberries. Strawberries, raspberries and blackberries are the most studied small fruit.

References

1. Rouseff, R., Leahy, M. M., Eds. *Fruit Flavors*; American Chemical Society: Washington, DC, 1995; p 292.
2. Zabetakis, I.; Holden, M. A. Strawberry flavour: Analysis and biosynthesis. *J. Sci. Food Agric.* **1997**, *74*, 421.
3. Harmon, A. D. In *Techniques for Analyzing Food Aroma*; Marsili, R., Ed.; Marcel Dekker: New York, 1997; p 81.
4. Rega, B.; Fournier, N.; Guichard, E. Solid phase microextraction (SPME) of orange juice flavor: Odor representativeness by direct gas chromatography olfactometry (D-GC-O). *J. Agric. Food Chem.* **2003**, *51*, 7092.
5. Nongonierma, A.; Cayot, P.; Quere, J. L.; Springett, M.; Voilley, A. Mechanisms of extraction of aroma compounds from foods, using adsorbents. Effect of various parameters. *Food Rev. Int.* **2006**, *22* (1), 51.
6. Roberts, D. D.; Pollien, P.; Milo, C. Solid-phase microextraction method development for headspace analysis of volatile flavor compounds. *J. Agric. Food Chem.* **2000**, *48*, 2430.
7. Williams, A.; Ryan, D.; Olarte, A.; Marriott, G. P.; Eddie, P. Analysis of strawberry volatiles using comprehensive two-dimensional gas chromatography with headspace solid-phase microextraction. *J. Chromatog.* **2005**, *817* (1), 97.
8. Honkanen, E.; Hirvi, T. In *Food Flavours*; Morton, I. D., MacLeod, A. J., Eds.; Elsevier Scientific Publications: Amsterdam, 1990; p 125.
9. Latrasse, A. In *Volatile Compounds in Foods and Beverages*; Maarse, H., Ed.; Dekker: New York, 1991; p 329.

Downloaded by 89.163.35.42 on June 23, 2012 | http://pubs.acs.org
Publication Date (Web): March 18, 2010 | doi: 10.1021/bk-2010-1035.ch001

10. Schieberle, P.; Hofmann, T. Evaluation of the character impact odorants in fresh strawberry juice by quantitative measurements and sensory studies on model mixtures. *J. Agric. Food Chem.* **1997**, *45*, 227.
11. Pyysalo, T.; Honkanen, E.; Hirvi, T. Volatiles of wild strawberries, *Fragaria vesca* L. compared to those of cultivated berries, *Fragaria* x *ananassa* cv. Senga Sengana. *J. Agric. Food Chem.* **1979**, *27*, 19.
12. Larsen, M.; Poll, L. Odor thresholds of some important aroma compounds in strawberries. *Z. Lebensm. Unters. Forsch.* **1992**, *195*, 120.
13. Aharoni, A.; Keizer, L. C.; Bouwmeester, H. J. Identification of the SAAT gene involved in strawberry flavour biogenesis by use of DNA microarrays. *Plant Cell* **2000**, *12*, 647.
14. Aharoni, A.; Giri, A. P.; Verstappen, F. W.; Bertea, C. M.; Sevenier, R.; Sun, Z.; Jongsma, M. A.; Schwab, W.; Bouwmeester, H. J. Gain and loss of fruit flavour compounds produced by wild and cultivated strawberry species. *Plant Cell* **2004**, *16*, 3110.
15. Raab, T.; Lopez-Raez, J. A.; Klein, D.; Caballero, J. L.; Moyano, E.; Schwab, W; Munoz-Blanco, J. FaQR, required for the biosynthesis of the strawberry flavor compound 4-hydroxy-2,5-dimethyl-3(2H)-furanone, encodes an enone oxidoreductase. *Plant Cell* **2006**, *18* (4), 1023.
16. Schwab, W.; Lunkenbein, S.; Salentijn, E. M. J.; Aharoni, A. In *Developments in Food Science*; Bredie, W., Petersen, M., Eds.; Elsevier B.V.: Amsterdam 2006, 43, p 39.
17. Zabetakis, I. In *Frontiers of Flavour Science*; Schieberle P., Engel, K.-H., Eds.; Deutsche Forschungsanstalt fuer Lebensmittelchemie: Garching, 2000, p 469.
18. Schwab, W.; Beuerle, T.; Roscher, R. In *Natural Product Analysis: Chromatography, Spectroscopy, Biological Testing*; Schreier P., Ed.; Vieweg: Wiesbaden, 1998, p 289.
19. Lavid, N.; Schwab, W.; Kafkas, E.; Koch-Dean, M.; Bar, E.; Larkov, O.; Ravid, U.; Lewinsohn, E. Aroma biosynthesis in strawberry: S-Adenosylmethionine:furaneol *O*-methyltransferase activity in ripening fruits. *J. Agric. Food Chem.* **2002**, *50*, 4025.
20. Wein, M.; Lavid, N.; Lunkenbein, S.; Lewinsohn, E.; Schwab, W.; Kaldenhoff, R. Isolation, cloning and expression of a multifunctional *O*-methyltransferase capable of forming 2,5-dimethyl-4-methoxy-3(2H)-furanone, one of the key aroma compounds in strawberry fruits. *Plant J.* **2002**, *6*, 755.
21. Sanz, C.; Perez, A. G.; Olias, R. Effects of temperature on flavor components in "in vitro" grown strawberry. *Acta Hort.* **2002**, *567* (1), 365.
22. Perez, A. G.; Sanz, C. Effect of high-oxygen and high-carbon-dioxide atmospheres on strawberry flavor and other quality traits. *J. Agric. Food Chem.* **2001**, *49* (5), 2370.
23. Larsen, M.; Watkins, C. B. Firmness and concentrations of acetaldehyde, ethyl acetate and ethanol in strawberries stored in controlled and modified atmospheres. *Postharvest Biol. Technol.* **1995**, *5*, 39.
24. Watson, R.; Wright, C. J.; McBurney, T.; Taylor, A. J.; Linforth, R. S. T. Influence of harvest date and light integral on the development of strawberry flavour compounds. *J. Exp. Bot.* **2002**, *53* (377), 2121.

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Publication Date (Web): March 18, 2010 | doi: 10.1021/bk-2010-1035.ch001

25. Cockshull, K. E.; Graves, C. J.; Cave, C. R. J. The influence of shading on yield of glasshouse tomatoes. *J. Hort. Sci.* **1992**, *67*, 11.
26. Wrolstad, R. E.; Culbertson, J. D.; Nagaki, D. A.; Madero, C. F. Sugars and nonvolatile acids of blackberries. *J. Agric. Food Chem.* **1980**, *28*, 553.
27. Plowman, J. E. Sugars and acids of raspberries, blackberries, and other brambles. *Lebensm.-Wiss. Technol.* **1991**, *24*, 113.
28. Scanlan, R. A.; Bills, D. D.; Libbey, L. M. Blackberry flavor components of commercial essence. *J. Agric. Food Chem.* **1970**, *18*, 744.
29. Houchen, M.; Scanlan, R. A.; Libbey, L. M.; Bills, D. D. Possible precursor for 1-methyl-4-isopropenylbenzene in commercial blackberry flavor essence. *J. Agric. Food Chem.* **1972**, *20*, 170.
30. Gulan, M. P.; Veek, M. H.; Scanlan, R. A.; Libbey, L. M Compounds identified in commercial blackberry essence. *J. Agric. Food Chem.* **1973**, *21*, 741.
31. Nijssen, L. M., Visscher, C. A., Maarse, H., Willemsens, L. C., Boelens, M. H., Eds. In *Volatile Compounds in Food, Qualitative and Quantitative Data*, 7th ed.; TNO Nutrition and Food Research Institute: Zeist, The Netherlands, 1996, p 15.1.
32. Turemis, N.; Kafkas, E.; Kafkas, S.; Kurkcuoglu, M.; Baser, K. H. C. Determination of aroma compounds in blackberry by GC/MS analysis. *Chem. Natural Compd.* **2003**, *39* (2), 174.
33. Klesk, K; Qian, M. Preliminary aroma comparison of Marion (*Rubus* spp. *hyb*) and Evergreen (*R. laciniatus* L.) blackberries by dynamic headspace/OSME technique. *J. Food Sci.* **2003a**, *68* (2), 697.
34. Klesk, K.; Qian, M. Aroma extract dilution analysis of cv. Marion (*Rubus* spp. *hyb*) and cv. Evergreen (*R. laciniatus* L.) blackberries. *J. Agric. Food Chem.* **2003b**, *51* (11), 3436.
35. Qian, M. C.; Wang, Y. Seasonal variation of volatile composition and odor activity value of 'Marion' (*Rubus* spp. *hyb*) and 'Thornless Evergreen' (*R. laciniatus* L.) berries. *J. Food Sci.* **2005**, *70* (1), C13.
36. Wang, Y.; Finn, C.; Qian, M. C. Impact of growing environment on chickasaw berry (*Rubus* L.) aroma evaluated by gas chromatography olfactometry dilution analysis. *J. Agric. Food Chem.* **2005**, *53*, 3563.
37. Klesk, K.; Qian, M.; Martin, R. R. Aroma extract dilution analysis of cv. Meeker (*Rubus idaeus* L.) red raspberries from Oregon and Washington. *J. Agric. Food Chem.* **2004**, *52*, 5155.
38. Malowicki, S. M. M.; Martin, R.; Qian, M. C. Volatile composition in raspberry cultivars grown in the Pacific Northwest determined by stir bar sorptive extraction-gas chromatography-mass spectrometry. *J. Agric. Food Chem.* **2008**, *56*, 4128.
39. Malowicki, S. M. M.; Martinm, R; Qian, M. C. Comparison of sugar, acids, and volatile composition in raspberry bushy dwarf virus-resistant transgenic raspberries and the wild type 'Meeker' (*Rubus Idaeus* L.). *J. Agric. Food Chem.* **2008**, *56*, 6648.

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Chapter 2

Evaluating Health Benefits of Various Fruits

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Fruits are an essential part of our daily diets. Most fruits are naturally low in fat, sodium, and calories. Fruits are important sources of many nutrients, including potassium, dietary fiber, vitamin C and folic, acid and they do not contain cholesterol. Some fruits have laxative effect, and prevent urinary tract infections or kidney stone formation. Phytonutrients in fruits have been shown to stimulate natural detoxifying enzymes in the body and lower the risk of atherosclerosis and cancer. Fruit consumption in US is less than half of the daily recommended level. Comprehensive national efforts are needed to increase fruit consumption for school children, adolescents and adults. Stock up fruits and never be fruitless! Bag some fruit for your morning commute. Buy fresh fruits in season when they taste best and cost less. The recommended consumption of fruits is 1 cup/1000 Calories or 2-2.5 cups (400-500g) per day. Fruit consumption needs to be doubled and it would improve nutrition and lower the risk of premature degenerative diseases.

Consumption of fruits as a significant portion of our daily diets has been associated with a lower risk of coronary heart disease and cancer (*1–3*). The USDA Food and Nutrition Information Center (2005) Food Guide Pyramid -Steps to a Healthier You (*4*) recommends daily active life, intake of low fat food products and consumption of vegetables and fruits. Food guide recommends consuming 1 cup/1000 calories or 2-2.5 cups (400-500g) of fruits every day. Most fruits are naturally low in fat, sodium, and calories. Fruits are important sources of many nutrients, including potassium, dietary fiber, vitamin C, folic acid and they

In Flavor and Health Benefits of Small Fruits; Qian, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

do not contain cholesterol. Some of the fruits listed by the USDA food pyramid include apples, apricots, avocado, bananas, blueberries, cantaloupe, cherries, grapefruit, guava, grapes, honeydew, kiwi fruit, lemons, limes, loquat, leeches, mangoes, nectarines, oranges, papaya, peaches, pears, pineapple, plums, prunes, raisins, raspberries, strawberries, tangerines and watermelon. It also includes fruit cocktail and 100% fruit juice. Phytonutrients in these fruits have been shown to stimulate natural detoxifying enzymes in the body and lower the risk of atherosclerosis and cancer (*5*). Prevention of urinary tract infection and kidney stone formation with cranberry juice has been reported (*6*, *7*). A laxative effect of prunes is believed to be due to its sorbitol, neochlorogenic and chlorogenic acids (*8*). Toxic metabolites in the gut and secondary bile acids increase the risk of colorectal cancer (*9*). Fruits are high in health promoting phytonutrients; blueberries, plums, prunes, strawberries, cranberries and cherries are rich in antioxidants, hydroxycinnamic acids, flavonoids (anthocyanins, flavonols, and proanthocyanidins), while apples contain polyphenols and catechins (*10*, *11*).

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Evaluating *in Vitro* Bile Acid Binding as Potential Health Benefits of Various Fruits

In vitro bile acid binding without the use of labeled isotopes is an economical method for screening various foods and food fractions to evaluate their healthful potential before initiating time and cost intensive animal and human studies. Bile acids are acidic steroids synthesized in the liver from cholesterol. After conjugation with glycine or taurine, they are secreted into the duodenum. Bile acids are actively reabsorbed by the terminal ileum and undergo an enterohepatic circulation (*12*). The bile acids are needed for the absorption of dietary fat from the GI tract. The dietary fat is metabolized to acetate. Acetate is the principal precursor of cholesterol synthesis in the body. Binding of bile acids and increasing fecal excretion has been hypothesized as a possible mechanism for lowering cholesterol by dietary fiber (*13–15*). By binding bile acids, food fractions prevent their reabsorption and stimulate plasma and liver cholesterol conversion to additional bile acids (*16–18*). Toxic metabolites in the gut and secondary bile acids increase the risk of colorectal cancer (*9*). The healthful, cholesterol-lowering (atherosclerosis amelioration, detoxification of harmful carcinogenic metabolites) potential of food fractions could be predicted by evaluating their in vitro bile acid binding, based on positive correlations found between in vitro and in vivo studies showing that cholestyramine (bile acid binding, cholesterol lowering drug) binds bile acids and cellulose does not (*19–22*). *In vitro* bile acid binding procedure in described in Figure 1.

Composition of various fruits tested for in vitro bile acid binding studies is given in Table I (*23*, *24*). On dry matter basis the fruits tested ranged in values for carbohydrates (82-96%), total dietary fiber (5-36%), sugar (31-87%), polysaccharides (5-90%), protein 2-10%, fat (1-4%) and minerals (1-6%).

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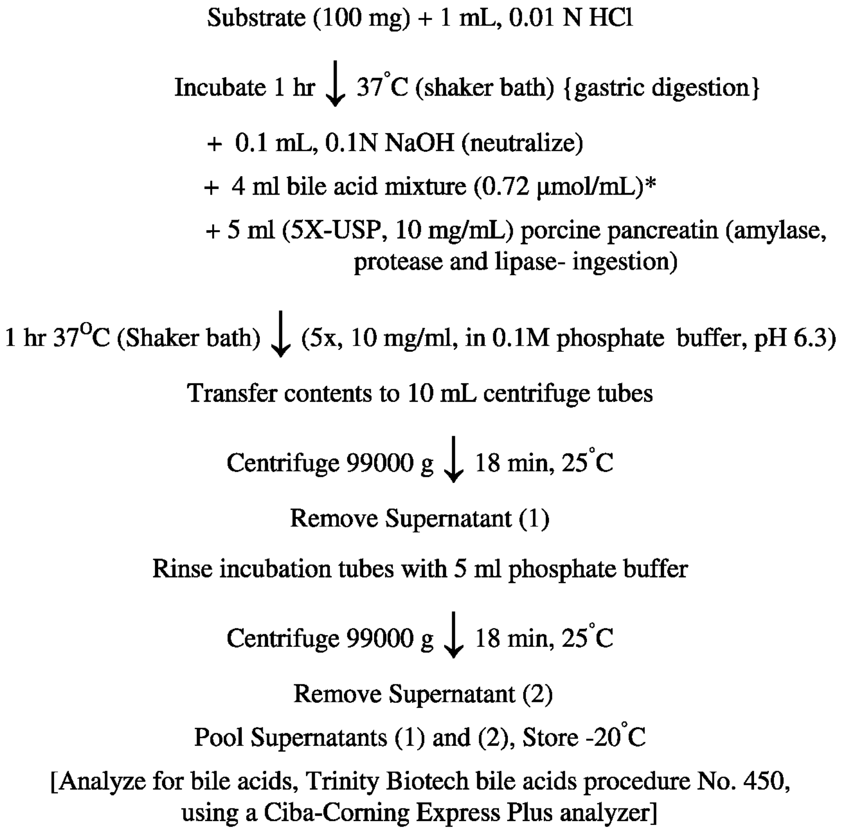

Substrate (100 mg) + 1 mL, 0.01 N HCl

Incubate 1 hr ↓ 37°C (shaker bath) {gastric digestion}

+ 0.1 mL, 0.1N NaOH (neutralize)

+ 4 ml bile acid mixture (0.72 μmol/mL)*

+ 5 ml (5X-USP, 10 mg/mL) porcine pancreatin (amylase, protease and lipase- ingestion)

1 hr 37°C (Shaker bath) ↓ (5x, 10 mg/ml, in 0.1M phosphate buffer, pH 6.3)

Transfer contents to 10 mL centrifuge tubes

Centrifuge 99000 g ↓ 18 min, 25°C

Remove Supernatant (1)

Rinse incubation tubes with 5 ml phosphate buffer

Centrifuge 99000 g ↓ 18 min, 25°C

Remove Supernatant (2)

Pool Supernatants (1) and (2), Store -20°C

[Analyze for bile acids, Trinity Biotech bile acids procedure No. 450, using a Ciba-Corning Express Plus analyzer]

*Figure 1. Diagram of the Bile Acid Binding Procedure (*0.1M phosphate buffer, pH 6.3 (phosphate buffer only for blank).*

In vitro bile acid binding of various fruits tested on dry matter basis is given in Table II. Relative to cholestyramine for bananas, blueberries, cherries, grapes, peaches, pears, pineapple, plums, prunes and strawberries the bile acid binding of 5–9% is very encouraging, which could be an indicator of their health promoting potential. Similarly 5–9% relative bile acid binding for oat bran and oat bran ready to eat cereal (cereal with US FDA approved for label health claim or lowering cholesterol) have been reported (*22*, *25*). These results (*23*, *24*) indicate that the relative health promoting potentials are: bananas = blueberries > peaches = plums = pineapple = prunes = strawberries ≥ grapes = pears > apricots > nectarines = apples, as indicated by their bile acid binding on a dry matter (dm) basis. The differences in bile acid binding between various fruits tested may relate to their phytonutrients, antioxidants, polyphenols, flavonoids (anthocyanins, flavonols, and proadnthocyanidins), structures, hydrophobicity of undigested fractions, anionic or cationic natures of the metabolites produced during digestion or their interaction with active binding sites. Inclusion of bananas, peaches, pineapple, grapes and pears in our daily diet, as health-promoting fruits should be encouraged. Animal studies are planned to explore relative healthful potential

Table I. Composition of apples (*Malus sylvestris*), apricots (*Prunus armeniaca*), bananas (*Musa paradisiaca*), blueberries (*Vaccinium* spp.), cherries (*Malpighia punicifolia*), cranberries (*Vaccinium macrocarpon*), grapes (*Vitis* spp.), nectarines (*Prunus persica*, *P. nectarina*), peaches (*Prunus persica*), pears (*Pyrus communis*), pineapple (*Ananus comosus*), plums (*Prunus* spp.), prunes (*Prunus* spp.), and strawberries (*Fragaria* x *ananassa*)*

Source	*Carb*[a]	*TDF*[a]	*Sugar*	*Plysc*[a]	*Prot*[a]	*Fat*	*Minerals*
Apples	95.6	16.6	72.0	23.7	1.8	1.2	1.3
Apricots	81.5	14.7	67.7	13.8	10.3	2.9	5.5
Bananas	91.0	10.4	48.7	42.3	4.3	1.3	3.3
Blueberries	91.8	15.2	63.1	28.7	4.7	2.1	1.5
Cherries	89.5	12.8	NL	89.5	4.7	3.5	2.3
Cranberries	94.8	35.7	31.4	63.4	3.0	1.0	1.2
Grapes	91.7	4.8	86.9	4.8	3.4	1.9	3.0
Nectarines	85.0	13.7	63.6	21.4	8.5	2.6	3.9
Peaches	85.7	13.5	75.4	10.3	8.2	2.2	3.9
Pears	94.9	19.0	60.2	34.7	2.3	0.7	2.0
Pineapple	93.3	10.3	68.4	24.9	4.0	0.9	1.8
Plums	89.4	11.0	77.7	11.7	5.5	2.2	2.9
Prunes	92.5	10.3	55.2	37.3	3.2	0.6	3.8
Strawberries	84.9	22.1	51.5	33.4	7.4	3.3	4.4

* Values are percent based on dry matter. [a] Carb = Carbohydrate; TDF = Total Dietary Fiber; Plysc = Polysaccharides; Prot = Protein. Polysaccharides = Carbohydrate – Sugar; NL, not listed. Data from refs (*23*) and (*24*).

for atherosclerosis amelioration (lowering lipids and lipoprotein) and cancer prevention (excretion of toxic metabolites, secondary bile acids) of the fruits studied.

Health Benefits of Consuming Fruits

Eight week feeding of diet rich in fruits and vegetables along with low total and saturated fat dairy products to hypertensive subjects with systolic (>140) diastolic (90) mm Hg blood pressure, resulted in the reductions in systolic (11.4) and diastolic (5.5) mm Hg (*26*). In individuals with base line systolic (131) and diastolic (85) mm Hg blood pressure reductions were systolic (5.5) and diastolic (3.0) mm Hg. For coronary heart disease nine of ten ecological studies, two of three case-control studies and six of 16 cohort studies found a significant protective association with consumption of fruit and vegetables (*3*). For stroke three of five ecological studies, six of eight cohort studies and for total circulatory

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disease, one of two cohort studies reported a significant protective association with consumption of fruit and vegetables.

After adjustment for standard cardiovascular risk factors, with the consumption fruits and vegetables, persons in the highest quintile intake had 20% lower relative risk for coronary heart disease compared with those in the lowest quintile of intake. Each 1-serving/d increase in intake of fruits or vegetables was associated with a 4% lower risk for coronary heart disease (*27*).

Mortality data over nineteen years of the NHANES subjects revealed that those consuming fruit and vegetables ≥ 3 times/d compared with <1 time/d had 27% lower stroke incidence, 42% lower stroke mortality, 24% lower ischemic heart disease mortality, 27% lower cardiovascular disease mortality, and 15% lower all-cause of mortality after adjustment for established cardiovascular disease risk factors (*28*).

Consumption of abundance of fruits and vegetables, whole grains as the main form of carbohydrate, non-hydrogenated unsaturated fats as the predominant form of dietary fat, and adequate omega-3 fatty acids can offer significant protection against coronary heart disease (CHD), together with regular physical activity, avoidance of smoking, and maintaining a healthy weight, may prevent the majority of cardiovascular disease in Western populations (*29*).

Higher fruit and vegetable intake was also associated with a lower risk of myocardial infarction, with an adjusted relative risk of 0.62 for extreme quintiles (*30*). Based on the third National Health and Nutrition Examination survey (NHANES III 1988-1994) the whites consumed significantly more servings of fruits and vegetables than did either black or Mexican Americans (*31*). Specifically, whites averaged 4.90±3.53 servings of fruits and vegetables/d, compared with 4.57±3.40 servings/d for Mexican Americans and 3.99±3.38 servings/d for blacks. The neighborhood socioeconomic status (NSES) was positively associated with fruit and vegetable intake: one standard deviation increase in NSES index was associated with consumption of nearly two additional servings of fruits and vegetables per week.

In Danish school children 11-, 13- and 15-year-old (Figure 2a, 2b, 2c) analyses of long-term trends in prevalence of those who ate fruit at least once daily suggest that in all age and gender groups, intake decreased from 1988 to 2002. Comprehensive nationwide initiatives to increase fruit and vegetable intake among Danish children and adolescents resulted in increase of fruit intake between 2002 and 2006. However, the situation in Denmark remains the same as in many other countries, where too many schoolchildren still do not meet the national recommendation for fruit intake (*32*).

Fruits and vegetable consumption in European countries during 1980 to 2003 is shown in Figure 3 (*33*). In Mediterranean countries fruits and vegetables consumption was higher. These counties are also associated with healthful Mediterranean diet. The fruit and vegetable consumption in US is similar to that of the United Kingdom. In order to reduce the risk of premature degenerative diseases there is essentially need to double fruit and vegetable consumption.

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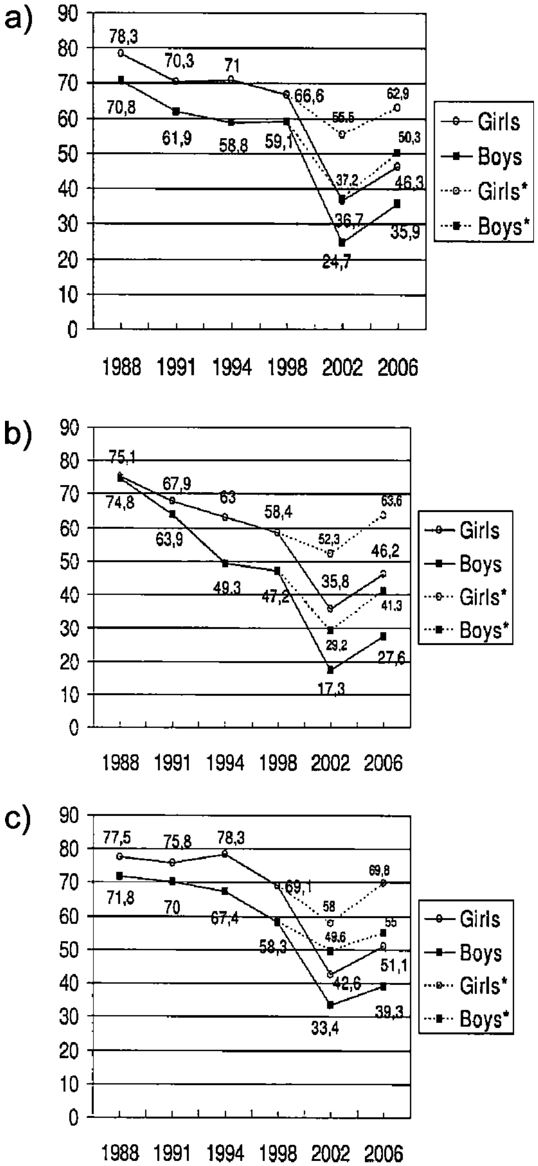

- *Alternative cut-point (methodological artefacts since the measurement of fruit intake changed twice over this 18-year period), n =23,871.
- Data from Rasmussen et al., 2008.

Figure 2. Percent (a) 11-Year-Olds, (b) 13-Year-Olds, and (c) 15-Year-Olds Eating Fruit at Least Once Per Day.

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Table II. In vitro bile acid binding by apples (*Malus sylvestris*), apricots (Prunus armeniaca), bananas (*Musa paradisiaca*), blueberries (*Vaccinium* spp.), cherries (*Malpighia punicifolia*), cranberries (*Vaccinium macrocarpon*), grapes (*Vitis* spp.), nectarines (*Prunus persica*, *P. nectarina*), peaches (*Prunus persica*), pears (*Pyrus communis*), pineapple (*Ananus comosus*), plums (*Prunus* spp.), prunes (*Prunus* spp.), and strawberries (*Fragaria* x *ananassa*) on equal weight, dry matter basis[ab]

Treatment	*Bile acid binding (µ mol/100 mg dm)*	*Bile cid binding relative to Cholestyramine, %*
Apples	0.12 ± 0.01ef	1.2 ± 0.1ef
Apricots	0.31 ± 0.02e	3.0 ± 0.2e
Bananas	0.90 ± 0.04b	8.8 ± 0.4b
Blueberries	0.73 ± 0.02b	7.1 ± 0.2b
Cherries	0.49 ± 0.03d	4.8 ± 0.3d
Cranberries	0.43 ± 0.04d	4.1 ± 0.4d
Grapes	0.50 ± 0.02d	4.9 ± 0.2d
Nectarines	0.21 ± 0.02e	2.1 ± 0.2e
Peaches	0.60 ± 0.03c	5.8 ± 0.3c
Pears	0.47 ± 0.01d	4.5 ± 0.1d
Pineapple	0.59 ± 0.02c	5.7 ± 0.2c
Plums	0.60 ± 0.01c	5.8 ± 0.1c
Prunes (plums, dried)	0.53 ± 0.06cd	5.1 ± 0.6cd
Strawberries	0.52 ± 0.03cd	5.1 ± 0.3cd
Cholestyramine	10.29 ± 0.05a	100.0 ± 0.4a
Cellulose	0.07 ± 0.02f	0.7 ± 0.2f

[a] Mean ± SEM; within a column different letters differ significantly ($P \leq 0.05$), n = 6.
[b] The dry matter used for incubation was 103–107 mg; cholestyramine and cellulose 24–26 mg. Data from refs (*23*) and (*24*).

Recommendations

Recommended Fruit Intake 1 cup/1000 Calories, 2-2.5 cups (400-500g) per day

a) Never be fruitless!
b) Stock up fruits.
c) Bag some fruit for your morning commute.
d) Buy fresh fruits in season when they taste best and cost less.

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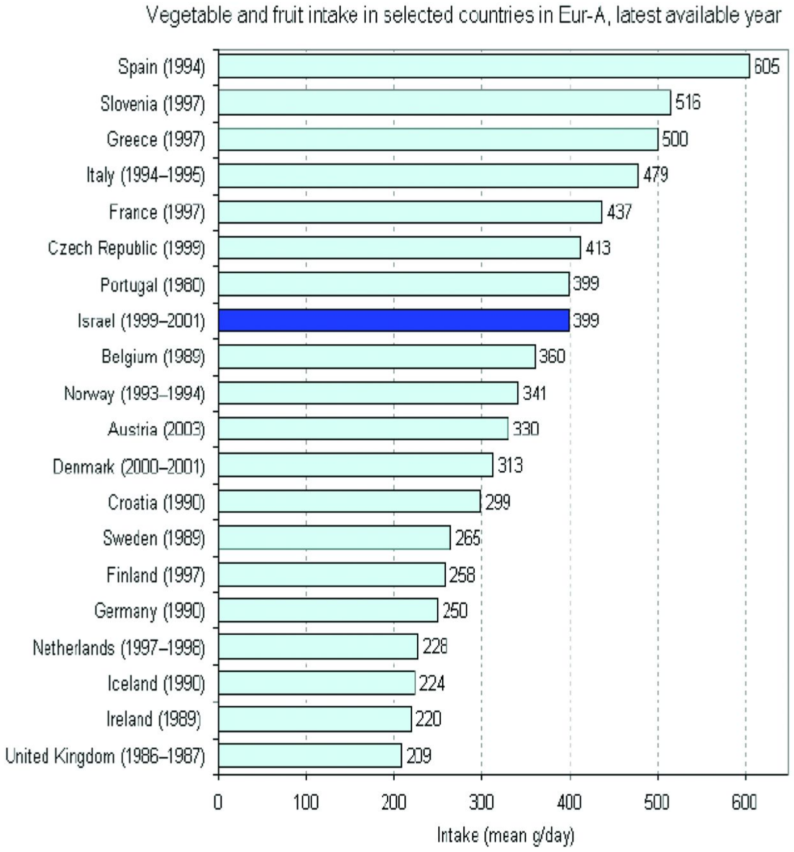

Vegetable and fruit intake in selected countries in Eur-A, latest available year

Sources: WHO Regional Office for Europe (2004a), Robertson et al. (2004) for data on Germany, Greece, Ireland and Spain, IFEW (2003) for data on Austria, Danish Institute of Food and Veterinary Research (2004) for data on Denmark and Israeli Center for Disease Control (2003) for data on Israel.

Figure 3. Fruit and vegetable consumption in European countries (see ref (33)).

Disclaimer

Names are necessary to report factually on available data; the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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References

1. Block, G.; Patterson, B.; Subar, A. Fruit, vegetables, and cancer prevention: A review of the epidemiological evidence. *Nutrition and Cancer - An International Journal* **1992**, *18*, 1–29.
2. Gaziano, J. M.; Manson, J. E.; Branch, L. G.; Colditz, G. A.; Willett, W. C.; Buring, J. E. A prospective study of consumption of carotenoids in fruits and vegetables and decreased cardiovascular mortality in the elderly. *Ann. Epidemiol.* **1995**, *5*, 255–260.
3. Ness, A. R.; Powles, J. W. Fruit and vegetables, and cardiovascular disease: A review. *Int. J. Epidemiol.* **1997**, *26*, 1–13.
4. USDA. Food and Nutrition Information Center Food Guide Pyramid. Steps to a Healthier You. http://www.mypyramid.gov (2005).
5. Ames, B. N.; Shigenaga, M. K.; Hagen, T. M. Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 7915–7922.
6. Kessler, T.; Jansen, B.; Hesse, A. Effect of blackcurrant-,cranberry- and plum juice consumption on risk factors associated with kidney stone formation. *Eur. J. Clin. Nutr.* **2002**, *56*, 1020–1023.
7. Kontiokari, T.; Sundqvist, K.; Nuutinen, M.; Pokka, T.; Koskela, M.; Uhari, M. Randomised trial of cranberry-lingonberry juice and Lactobacillus drink for the revention of urinary tract infections in women. *Br. Med. J.* **2001**, *322*, 1571–1573.
8. Stacewicz-Sapuntzakis, M.; Bowen, P. E.; Hussain, E. A.; Damayanti-Wood, B. I.; Farnsworth, N. R. Chemical composition and potential health effects of prunes: A functional food?. *Crit. Rev. Food Sci. Nutr.* **2001**, *41*, 251–286.
9. Costarelli, V.; Key, T. J.; Appleby, P. N.; Allen, D. S.; Fentiman, I. S.; Sanders, T. A. A prospective study of serum bile acid concentrations and colorectal cancer risk in post-menopausal women on the island of Guernsey. *Br. J. Cancer* **2002**, *86*, 1741–1744.
10. Reed, J. Cranberry flavonoids, atherosclerosis and cardiovascular health. *Crit. Rev. Food Sci. Nutr.* **2002**, *42*, 301–316.
11. Sun, J.; Chu, Y. F.; Wu, X.; Liu, R. H. Antioxidant and antiproliferative activities of common fruits. *J. Agric. Food Chem.* **2002**, *50*, 7449–7454.
12. Hofmann, A. F. The enterohepatic circulation of bile acids in man. *Clin. Gastroenterol.* **1977**, *6*, 3–24.
13. Trowell, H. C. *Refined Foods and Disease*; Burkitt, D. P., Trowell, H. C., Eds.; Academic Press: London, 1975; pp 195−226.
14. Lund, E. K.; Gee, J. M.; Brown, J. C.; Wood, P. J.; Johnson, I. T. Effect of oat gum on the physical properties of the gastrointestinal contents and on the uptake of D-galactose and cholesterol by rat small intestine in vitro. *Br. J. Nutr.* **1989**, *62*, 91–101.
15. Anderson, J. W.; Siesel, A. E. Hypocholesterolemic effects of oat products In *New Developments in Dietary Fiber: Physiological, Physiochemical, and Analytical Aspects*; Furda, I., Brine, C. J., Eds.; Plenum Press: New York, 1990; pp 17−36.

16. Eastwood, M. A.; Hamilton, D. Studies on the adsorption of bile acids to non-absorbed components of diet. *Biochim. Biophys. Acta* **1968**, *152*, 165–173.
17. Balmer, J.; Zilversmit, D. B. Effect of dietary roughage on cholesterol absorption, cholesterol turnover and steroid excretion in the rat. *J. Nutr.* **1974**, *104*, 1319–1328.
18. Kritchevsky, D.; Story, J. A. Binding of bile salts in vitro by nonnutritive fiber. *J. Nutr.* **1974**, *104*, 458–462.
19. Suckling, K. E.; Benson, G. M.; Bond, B.; Gee, A.; Glen, A.; Haynes, C.; Jackson, B. Cholesterol lowering and bile acid excretion in the hamster with cholestyramine treatment. *Atherosclerosis* **1991**, *89*, 183–190.
20. Nakamura, T.; Matsuzawa, Y. Drug treatment of hyperlipoproteinemia: Bile acid-binding resins. *Nippon Rinsho* **1994**, *52*, 3266–3270.
21. Daggy, B. P.; O'Connell, N. C.; Jerdack, G. R.; Stinson, B. A.; Setchell, K. D. Additive hypocholesterolemic effect of psyllium and cholestyramine in the hamster: influence on fecal sterol and bile acid profiles. *J. Lipid Res.* **1997**, *38*, 491–502.
22. Kahlon, T. S.; Chow, F. I. In vitro binding of bile acids by rice bran, oat bran, wheat bran and corn bran. *Cereal Chem.* **2000**, *77*, 518–521.
23. Kahlon, T. S.; Smith, G. E. In vitro binding of bile acids by blueberries (*Vaccinium spp.*), plums (*Prunus spp*), prunes (*Prunus spp*), strawberries (*Fragaria X ananassa*), cherries (*Malpighia punicifolia*), cranberries (*Vaccinium macrocarpon*) and apples (*Malus sylvestris*). *Food Chem.* **2007a**, *100*, 1182–1187.
24. Kahlon, T. S.; Smith, G. E. In vitro binding of bile acids by bananas, peaches, pineapple, grapes, pears, apricots and nectarines. *Food Chem.* **2007b**, *101*, 1046–1051.
25. Kahlon, T. S.; Woodruff, C. L. In vitro binding of bile acids by various ready to eat breakfast cereals. *Cereal Foods World* **2003**, *48*, 73–75.
26. Appel, L. J.; Moore, T. J.; Obarzanek, E.; Vollmer, W. M.; Svetkey, L. P.; Sacks, F. M.; Bray, G. A.; Vogt, T. M.; Cutler, J. A.; Windhauser, M. M.; Lin, P. H.; Karanja, N. A clinical trial of the effects of dietary patterns on blood pressure. *N. Engl. J. Med.* **1997**, *336*, 1117–1124.
27. Joshipura, K. J.; Hu, F. B.; Manson, J. E.; Stampfer, M. J.; Eric, B.; Rimm, E. B.; Speizer, F. E.; Colditz, G.; Ascherio, A.; Rosner, B.; Spiegelman, D.; Willett, W.C. The effect of fruit and vegetable intake on risk for coronary heart disease. *Ann. Intern. Med.* **2001**, *134*, 1106–1114.
28. Bazzano, L. A.; Jiang He, J.; Ogden, L. G.; Loria, C. M.; Vupputuri, S.; Myers, L.; Whelton, P. K. Fruit and vegetable intake and risk of cardiovascular disease in US adults: The first National Health and Nutrition Examination Survey Epidemiologic Follow-Up Study. *Am. J. Clin. Nutr.* **2002**, *76*, 93–99.
29. Hu, F. B.; Willett, W. C. Optimal diets for prevention of coronary heart disease. *JAMA, J. Am. Med. Assoc.* **2002**, *288*, 2569–2578.
30. Liu, S.; Manson, J. E.; Lee, I. M.; Cole, S. R.; Hennekens, C. H.; Willett, W. C.; Buring, J. E. Fruit and vegetable intake and risk of cardiovascular disease: The Women's Health Study. *Am. J. Clin. Nutr.* **2000**, *72*, 922–928.

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31. Dubowitz, T.; Heron, M; Bird, C. E.; Lurie, N.; Finch, B. K.; Basurto-Da′vila, R.; Hale, L.; Escarce, J. J. Neighborhood socioeconomic status and fruit and vegetable intake among whites, blacks, and Mexican Americans in the United States. *Am. J. Clin. Nutr.* **2008**, *87*, 1883–1891.
32. Rasmussen, M.; Krølner, R.; Svastisalee, C. M.; Due, P.; Holstein, B. E. Secular trends in fruit intake among Danish schoolchildren, 1988 to 2006: Changing habits or methodological artefacts?. *Int. J. Behavioral Nutr. & Physical Activity* **2008**, *5*, 1–9.
33. World Health Organization 2006. Highlights on health in Israel 2004. http://www.euro.who.int/highlights.

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Chapter 3

Flavor Chemistry of Small Fruits: Blackberry, Raspberry, and Blueberry

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The increased interest in the health benefits of berry fruits has promoted research in horticulture and food science to address the fruit quality, especially in flavor quality. Flavor in fruit is largely determined by the content and variety of their particular volatile compounds. Volatiles found in fruits are diverse, consisting of hundreds of different chemical compounds, giving unique flavors to different fruits. The flavor of small fruits other than winegrapes has not been as well studied as other fruits such as tree fruits. Although the environment alters the flavor quality of fruit, genetic factors determine the flavor profile quantitatively and qualitatively. More studies are needed to understand the biogenesis of flavor compounds so that genomic tools related to flavor formation in small fruits can be developed and utilized. This type of information could be used in the future in the breeding program to develop new cultivar with desired flavor quality.

Introduction

The word ‘berry’ has two meanings: one, a botanical definition, the other, a common identification. Botanical berries are a simple fruit, having seeds and pulp produced from a single ovary, such as tomato, grape, lychee, loquat, lucuma, plantain, avocado, persimmon, eggplant, guava, uchuva, and chili pepper. In common language, however, berries are more broadly recognized as small, round or semi-oblong, usually brightly colored, sweet or sour fruit,

In Flavor and Health Benefits of Small Fruits; Qian, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

such as strawberry, raspberry, blackberry, blackcurrant, blueberry, cranberry, and elderberry (*1*). In North America, commonly consumed berry fruits include blackberries (*Rubus* spp.), black raspberries (*Rubus occidentalis*), blueberries (*Vaccinium corymbosum*), cranberries (*Vaccinium macrocarpon*), red raspberries (*Rubus idaeus*) and strawberries (*Fragaria* × *ananassa*) (*2*). Although there are many types of berry fruits consumed worldwide, this chapter focuses on three of the aforementioned berries commonly consumed in North America: red raspberries, blackberries, and blueberries.

Berry fruits are consumed not only in fresh and frozen forms, but also in a variety of foods such as juice, jams and jellies. In addition, the trend of using berry extracts as ingredients in functional foods and dietary supplements is growing. The nutritional content of berries includes dietary fiber, vitamins, minerals, and high levels of phenolic compounds. The major phenolic constituents in berries include anthocyanins, flavonols, flavanols, ellagitannins, gallotannins, proanthocyanidins, and phenolic acids (*3*). These phenolic compounds have been found to have various biological properties such as antioxidant, anticarcinogenic, anti-neurodegenerative, and anti-inflammatory activities (*3*). The consumption of berries in fresh, processed, and the nutraceutical markets is expanding exponentially in the last decades.

Nutritional value aside, berry flavor is of great importance to the consumer. Studies on berry flavor have been conducted over the past fifty years. Investigations in red raspberry flavor have been extensive, while studies on blackberry and blueberry flavor are still very limited. A quick scan of these studies shows that a large number of volatile compounds have been identified in berries. So far, approximately 250 volatile compounds have been identified in raspberries and blackberries, while about 100 volatile compounds have been identified in blueberries.

Some results were discrepant, and dependant on flavor isolation and identification techniques used; some compounds were not conclusive and were only identified in a single study. Traditional isolation technique, such as distillation, liquid-liquid extraction and headspace sampling, were frequently used in those studies to isolate flavor compounds from berry samples. However, many of these traditional techniques have disadvantages of bias, artifact formation and solvent contamination. Reliable quantitative data of volatile composition in berry samples, and sensory data in berries are very limited.

The flavor composition of berries is diverse. These compounds are enzymatically produced from lipids, carbohydrates, proteins, and amino acids in the fruits. Although the metabolic pathways might be similar in berries, each berry cultivar has a unique metabolic profile, this unique metabolic profile is responsible for the generation of unique volatile profile of each species, resulting in the characteristic aroma of the fruits. This characteristic aroma is the expression of the genotype-environment interaction. Typically, genetics determine the precursors, enzyme systems, and their activity in flavor formation. It is common that varietal differences in flavor are due to quantitative differences in flavor composition, rather than qualitative differences. The quantitative differences can also arise from environmental factors such as geography, soil nutrition, weather, irrigation and many other agronomical conditions.

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Reviews on the raspberry, blackberry, and blueberry flavor are available in references (*1*, *4*). Because of the growing number of studies in this field, this chapter provides an update of berry flavor research.

Raspberry

Wild and cultivated raspberries are in the subgenus *Idaeobatus* of the genus *Rubus*. The main types of raspberries are red raspberry and black raspberry. Others, such as yellow raspberries, result from a mutation of red raspberries that prevents the red color from forming, are grown in the same way as red raspberries. Purple raspberries are a hybrid between black and red raspberries. These species are, however, of little to no economic importance. The red raspberries are the most widely grown species.

Volatile compounds in raspberry have been studies for over fifty years, including wild selections, hybrid between different species, and multiple commercial cultivars (*5–8*). In addition, red raspberry juice, essential oils, and raspberry processed products have also been studied (*9–13*). More than 250 volatile compounds have been identified. The volatile profiles of red raspberries were dominated by norisoprenoids, lactones, carbonyl compounds, esters, and alcohols. The most abundant compounds reported in raspberries include α-ionone, β-ionone, raspberry ketone, linalool, geraniol, benzaldehyde, α-pinene, β-caryophyllene, β-myrcene, trans-β-ocimene, ethyl acetate, ethyl heptanoate, and 2-methylbutanol (*6–8*, *10*, *14*, *15*).

Gas chromatography-olfactometry (GC-O) technique has been used to determine the aroma-active compounds in raspberries. Major compounds in raspberries are summarized in Table 1. However, qualitative and quantitative studies on aroma contributing compounds are still limited. Klesk and Qian (*10*) studied the odor-active compounds in ‘Meeker’ raspberries from Oregon and Washington by Aroma Extract Dilution Analysis (AEDA) and GC/O-MS technique. Seventy-five aromas were identified in the red raspberry fruit. According to flavor dilution (FD) factors, furaneol, hexanal, α-ionone, β-ionone, 4-oxo-β-ionone, neo-allo-ocimene, β-pinene, β-damascenone, *cis*-3-hexenal, methional, *cis*-3-hexenol, linalool, butanoic acid, ethyl 2-methylpropanoate, *trans*-2-hexenal, 2, 3-butanedione, heptanal, thiophene, benzaldehyde, geraniol, and raspberry ketone, were considered very important to raspberry aroma. Roberts and Acree (*13*) studied the top odor-active compounds in fresh ‘Heritage’ raspberries using headspace analysis and solvent extraction techniques. They find that β-damascenone, diacetyl, sotolon, 1-hexen-3-one, and *cis*-3-hexenol,ethyl 2-methylbutanoate, ethyl butanoate, raspberry ketone, vanillin, *cis*-3-hexenal, and β-ionone are the most important aroma compounds in raspberry.

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Table 1. Major aroma compounds identified in red raspberries

Compounds	*References*	*Compounds*	*References*
Esters		*Alcohols*	
Ethyl acetate	(*5*, *6*, *10*, *20*, *23*)	1-Hexanol	(*5*, *6*, *10*)
Ethyl butanoate	(*10*, *13*)	*trans*-3-Hexenol	(*5*)
Ethyl 2-methyl-propanoate	(*10*)	*cis*-3-Hexenol	(*5–7*, *10*, *23*)
Ethyl 2-methylbutanoate	(*10*, *13*)	trans-2-Hexenol	(*5*)
Butyl acetate	(*10*)	1-Octen-3-ol	(*10*)
Ethyl propanoate	(*10*)	1-Octanol	(*10*)
3-Methylbutyl acetate	(*10*)	2-Nonanol	(*10*)
Methyl hexanoate	(*10*, *20*)		
Ethyl hexanoate	(*10*, *20*)	*Aromatic compounds*	
Ethyl octanoate	(*10*)	Benzyl alcohol	(*5–7*, *10*)
cis-3-Hexyl acetate	(*5*, *10*, *23*)	Phenylethyl alcohol	(*5*, *6*, *10*)
		Benzaldehyde	(*5*, *6*, *10*, *20*, *23*)
Terpenoids		Eugenol	(*5*, *10*)
α-Pinene	(*5*, *10*, *20*, *23*, *24*)	Vanillin	(*5*, *10*, *13*)
β-Pinene	(*10*, *20*, *24*)	Gingerone	(*5*, *10*)
α-Phellandrene	(*5*, *10*, *23*, *24*)	Raspberry ketone	(*5*, *7*, *10*, *13*)
β-Myrcene	(*10*, *23*)		
Limonene	(*5*, *10*, *20*, *23*)	*Acids*	
trans-β-Ocimene	(*10*)	Acetic acid	(*5–7*, *10*)
Sabinene	(*5*, *10*)	Propanoic acid	(*5*, *6*)
Caryophyllene	(*5*, *10*, *24*)	Butanoic acid	(*5*, *6*, *10*)
Linalool	(*5–7*, *10*, *23*, *24*)	2-Methylbutanoic acid	(*5*, *6*, *10*)
4-Terpineol	(*5*)	Hexanoic acid	(*5–7*)
α-Terpineol	(*5*, *10*)	Octanoic acid	(*5*, *6*)
Nerol	(*5*)		
Geraniol	(*5–7*, *10*)	*Lactones*	
		γ-Hexalactone	(*5*, *6*)

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Table 1. (Continued). Major aroma compounds identified in red raspberries

Compounds	*References*	*Compounds*	*References*
Carbonyl compounds		δ-Hexalactone	(*5*)
Hexanal	(*5*, *10*, *20*, *23*)	γ-Octalactone	(*5*, *6*)
Heptanal	(*10*, *23*)	δ-Octalactone	(*5*, *10*)
Octanal	(*10*, *13*, *20*, *23*)	δ-Decalactone	(*5*, *10*)
cis-3-Hexenal	(*10*, *13*)	δ-Dodecalactone	(*5*)
trans-2-Hexenal	(*5*, *10*, *23*)		
Diacetyl	(*6*, *10*, *13*)	*Miscellaneous*	
Acetoin	(*5–7*)	Mesifurane	(*5*)
2-Heptanone	(*10*, *20*)	Norfuraneol	(*5*)
2-Nonanone	(*10*)	Furaneol	(*5*, *10*, *13*)
2-Undecanone	(*10*)	Homofuraneol	(*10*)
		Sotolon	(*10*, *13*)
Norisoprenoids		Maple furanone	(*10*)
β-Damascenone	(*5*, *6*, *10*, *13*)		
α-Ionone	(*5–7*, *10*, *24*)	*Others*	
α-Ionol	(*10*)	Dimethyl sulfide	(*10*)
β-Ionone	(*6*, *7*, *10*, *13*, *20*, *24*)	Dimethyl disulfide	(*10*)
β-Dihydroionone	(*10*)	Methional	(*10*)

Raspberry ketone is considered to be the characteristic compound for raspberry aroma. Raspberry ketone was first isolated from raspberries by TLC and identified by Schinz & Seidel (*16*). Braun & Hieke (*17*), Gallois (*18*), and Maquin, et al, (*19*) have also examined this compound. Organoleptic evaluation of the raspberry fruits shows that increasing raspberry flavor is in agreement with increasing raspberry ketone content in the fruits. The raspberry aroma was found to depend on a relatively high raspberry ketone content (*7*, *14*, *15*).

The importance of other aroma compounds-- α-ionone, β-ionone, *cis*-3-hexenol, geraniol, linalool, benzyl alcohol, acetoin, acetic acid, and hexanoic acid--were also examined in different cultivars. Raspberry ketone, α-ionone, and β-ionone were found to be the most important aroma compounds in all raspberries regardless of the cultivar, while linalool and geraniol were found in considerable levels only in some cultivars.

The concentration of these aroma compounds are affected by genotype, fruit maturity, and many other factors. Differences in volatile composition are apparent among raspberry cultivars. Vereshchagin and Bezzubov (*11*) compared the cultivars ‘Newburgh’ and ‘Novost Kuzmina’ for volatile composition.

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‘Novost Kuzmina’ had greater quantities of alcohols and carbonyl compounds, whereas ‘Newburgh’ had more terpenoid compounds. The variation in raspberry ketone, α-ionone and β-ionone concentration among ten raspberry cultivars: ‘Andenken an Paul Camenzind’, ‘Chilcotin’, ‘Glen Clova’, ‘Glen Moy’, ‘Glen Prosen’, ‘Meeker’, ‘Rutrago’, ‘Skeena’, ‘Veten’, and ‘Zenith’, has been reported. Although the raspberry ketone concentration varied from 1 ppm to 4 ppm, minimal variation was observed for other compounds (*7*). Borejsza-Wysocki, et al (*15*), also reported variation of raspberry ketone among different cultivars. Cultivars such as ‘Canby’ and ‘Royalty’ contained less than 30 ppb of raspberry ketone, while ‘Willamette’ contained over 170 ppb. The content of raspberry ketone was also investigated on other cultivars. ‘Chilliwack’, ‘Comox’, ‘Malahat’, ‘Meeker’, ‘Tulameen’, and ‘Willamette’ had raspberry ketone concentrations varying from 0.01-0.05 mg/mL (*20*). More recently, Malowicki and Qian (*21*) quantified 29 volatile aroma compounds in ‘Chilliwack’, ‘Tulameen’, ‘Willamette’, ‘Yellow Meeker’, and ‘Meeker’ and find that volatile variation depends on specific compounds and cultivars. Large variations for α-ionone, β-ionone, geraniol, linalool, and *cis*-3-hexenol were observed in different raspberry cultivars.

Growing conditions will also cause flavor variations. Significant year-to-year differences in raspberry ketone, alcohol, aldehyde, ester, ketone, and terpene concentrations have been noticed in raspberries grown on the same plot, as studied by Moore (*20*). These variations were also witnessed between plots of raspberries, although the reported differences were generally smaller than the differences reported from year-to-year. Malowicki and Qian (*21*) also reported that the volatile composition of ‘Meeker’ raspberries grown in different locations varied. Raspberries grown in Oregon appeared to have higher concentration of δ-octalactone, δ-decalactone, geraniol, and linalool than the berries grown in Washington. It is not clear what agronomical parameter(s) causes these differences.

Flavor composition in fruits is dramatically influenced by the degree of ripening. Aroma volatiles generally change at the final stages of ripening. Guichard (*22*) reported dramatic increases in terpenoids during ripening and some increases in esters, although specific compounds in different cultivars responded differently in the overripe phase. A clear difference was shown between maturity stages for green-pink stage, pink stage and ripe/overripe stages; however, the flavor changes occurring as fruit reaches the overripe stage are not as evident as those that occur before the ripe stage. During the ripening of ‘Glen Prosen’ raspberries, the abundances of several monoterpenes including camphene, β-myrcene, and limonene rise steadily, as do the compounds α-phellandrene, α-pinene, α-ionone, β-ionone, methyl acetate, ethyl acetate, 2-methyl-1-butanol, and *cis*-3-hexenol. In addition, the concentration of volatiles associated with green leafy aromas declined (*23*).

The volatile aroma composition changes during the freezing process and long-term frozen storage are minimal. A significant increase was observed only for α-ionone and caryophyllene in some cultivars during long term (12 months) of storage (*24*).

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Blackberry

Blackberries, like raspberries, belong in the family *Rosaceae*, genus *Rubus*. Blackberry cultivars can be classified according to their growth habit: trailing, semi-erect, or erect. Blackberries are grown all over the world, and the Pacific Northwest of America is a leading production region. The major blackberries in this region are trailing types blackberries such as 'Thornless Evergreen', 'Marion', and 'Black Diamond'. Historically 'Marion' has been the most appreciated blackberry due to its unique aroma and flavor. However, with more acreage of new cultivars such as 'Black Diamond' being harvested this may change.

More than 250 volatile compounds have been identified in blackberry whole fruit, essence, juice, and other products (*25–28*). Volatile profiles of blackberry are diverse, including esters, aldehydes, ketones, lactones, terpenoids, norisoprenoids, alcohols, phenolics, furanone, and acids. The major volatiles identified in blackberry are summarized in Table 2.

Table 2. Major aroma compounds identified in blackberries

Compounds	*References*	*Compounds*	*References*
Esters		*trans*-2-Hexenol	(*27*, *31*)
Ethyl acetate	(*27*, *30*, *31*, *33*)	2-Heptanol	(*27*, *30*, *31*, *33*)
Ethyl 2-methylpropanoate	(*30*, *33*)	1-Octen-3-ol	(*27*, *30*)
Methyl butanoate	(*27*, *30*, *33*)	1-Octanol	(*27*, *30*, *31*, *33*)
Ethyl butanoate	(*27*, *30*, *33*)		
Ethyl 2/3-methylbutanoate	(*27*, *30*, *31*, *33*)	*Aromatic compounds*	
Butyl acetate	(*27*, *30*, *31*, *33*)	Methyl salicylate	(*31*)
Methyl hexanoate	(*27*, *30*, *33*)	Benzyl alcohol	(*27*, *30*, *33*)
Ethyl hexanoate	(*27*, *30*, *31*)	Phenylethyl alcohol	(*27*, *30*, *33*)
Hexyl acetate	(*27*, *31*)	Cinnamaldehyde	(*27*, *30*)
		Eugenol	(*27*, *31*, *33*)
Terpenoids		Isoeugenol	(*27*)
α-Phellandrene	(*31*)	Vanillin	(*27*, *33*)
Myrcenene	(*30*, *33*)	Methyl vanillate	Unpublished
Limonene	(*30*, *31*, *33*)	Zingerone	Unpublished
Linalool	(*27*, *30*, *31*, *33*)	Raspberry ketone	Unpublished
4-Terpineol	(*27*, *31*, *33*)		
Borneol	(*27*, *31*)	*Acids*	

Continued on next page.

Table 2. (Continued). Major aroma compounds identified in blackberries

Compounds	*References*	*Compounds*	*References*
α-Terpineol	(*27*, *31*)	Acetic acid	(*27*, *30*, *31*, *33*)
Carvone	(*27*, *30*, *33*)	Butanoic acid	(*30*, *31*, *33*)
Myrtenol	(*27*, *31*, *33*)	2-methylbutanoic acid	(*30*, *31*, *33*)
Nopol	(*31*)	Hexanoic acid	(*27*, *30*, *31*, *33*)
Citronellol	(*31*, *33*)		
Nerol	(*33*)	*Lactones*	
Geraniol	(*27*, *33*)	γ-Octalactone	(*27*)
Perillyl alcohol	(*27*, *31*)	δ-Octalactone	(*27*)
		γ-Decalactone	(*27*)(*33*)
Carbonyl compounds		δ-Decalactone	(*27*)
3-Methylbutanal	(*27*, *30*, *31*)	γ-Undecalactone	(*27*)
Hexanal	(*27*, *30*, *31*, *33*)		
trans-2-Hexenal	(*27*, *31*, *33*)	*Miscellaneous*	
trans,*trans*-2,4-Heptadienal	(*27*, *31*)	*cis* / *trans*-Linalool oxide	(*30*, *31*)
Diacetyl	(*27*, *30*, *33*)	Theaspirane A / B	(*27*, *30*, *31*, *33*)
2-Heptanone	(*27*, *30*, *31*, *33*)	Mesifurane	(*27*)
1-Octen-3-one	(*30*)	Furaneol	(*30*, *33*, *45*)
2-Undecanone	(*27*, *30*, *31*, *33*)	Homofuraneol	(*30*, *33*)
		Sotolon	(*30*)
Norisoprenoids		Maple furanone	(*30*)
Damascenone	(*27*, *30*, *33*)		
α-Ionone	(*31*)	*Others*	
β-Ionone	(*27*, *31*, *33*)	Dimethyl sulfide	(*30*, *33*)
		Thiophene	(*30*)
Alcohols		2-Methylthiophene	(*30*)
1-Hexanol	(*27*, *30*, *31*)	Dimethyl disulfide	(*30*)
cis-3-Hexenol	(*27*, *31*, *33*)	Methional	(*30*, *33*)

The essence of ‘Thornless Evergreen’ blackberry was first investigated in the 1970s (*25*, *26*). Out of twenty-three volatile compounds identified, only 3, 4-dimethoxyallylbenzene was identified to contribute to the characteristic ‘Thornless Evergreen’ flavor. More intensive studies (*27*) of fresh ‘Thornless

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Evergreen' by fractionation and gas chromatography-olfactometry identified more than 200 compounds. The major aroma compounds were identified as 2-heptanol, *p*-cymen-8-ol, 2-heptanone, 1-hexanol, α-terpineol, pulegone, 1-octanol, isoborneol, myrtenol, 4-terpineol, carvone, elemicine, and nonanal. Mixing appropriate amounts of these compounds resulted in an odor somewhat reminiscent of blackberries, but lacking the delicate aroma of the natural extract.

The aroma compounds in 'Thornless Evergreen' and 'Marion' blackberries were investigated using Aroma Extract Dilution Analysis as well as odor-activity values (ratio of the concentration of a compound in the sample to its threshold) (*29–31*). According to flavor dilution factor (FD values), 2,5-dimethyl-4-hydroxy-3-(2H)-furanone, 2-ethyl-4-hydroxy-5-methyl-3-(2H)-furanone, 4-hydroxy-5-methyl-3-(2H)-furanone, 4,5-dimethyl-3-hydroxy-2-(5H)-furanone, and 5-ethyl-3-hydroxy-4-methyl-2-(5H)-furanone are prominent aromas in 'Thornless Evergreen' and 'Marion', however, the ratio is different for the two cultivars. Furaneol is very important to the aroma of both cultivars, however, 'Marion' has 16 times more furaneol than 'Thornless Evergreen' (*32*). Other major odorants in 'Thornless Evergreen' are ethyl hexanoate, 2-heptanone, ethyl 2-methylbutanoate, 2-heptanol, 3-methylbutanal, α-pinene, limonene, *p*-cymene, linalool, *trans*-2-hexenal, myrtenal, hexanal, 2-methylbutanal, and sabinene, while the other major aroma compounds in 'Marion' are ethyl hexanoate, ß-ionone, linalool, 2-heptanone, α-ionone, and hexanal (*31*).

As with many other fruits, blackberry flavor is affected by environmental factors. Seasonal variations always exist for agricultural products. Qian and Wang (*31*) analyzed the volatile compositions of 'Marion' and 'Thornless Evergreen' blackberries from three growing seasons. Seasonal variations were present for both cultivars, however, the variation and magnitude of change is highly dependent on the aroma compounds, and the cultivar difference is the determining factor for the difference in aroma composition.

Growing regions have a major impact on aroma composition. Wang *et al.* (*33*) compared the aroma profile of 'Chickasaw' blackberry growing in Oregon and Arkansas. The major aroma compounds in 'Chickasaw' blackberry grown in Oregon are ethyl butanoate, linalool, methional, *trans*, *cis*-2, 6-nonadienal, *cis*-1, 5-octadien-3-one, and 2, 5-dimethyl-4-hydroxy-3(2H)-furanone. In comparison, the major aroma compounds in Arkansas-grown berries were ethyl butanoate, linalool, methional, ethyl 2-methylbutanoate, β-damascenone, and geraniol. The higher level of *trans*, *cis*-2, 6-nonadienal and *cis*-1, 5-octadien-3-one is probably caused by lower mean growing temperature in Oregon during the ripening season of June and July. The ripening conditions in Oregon give fruit more "green" and "fruity" notes than berries grown in Arkansas.

Heating blackberry juice can dramatically alter the flavor profile (*28*, *34*). It has been found that the concentrations of aldehydes, lactones, and furan compounds increased significantly upon heating, and furfural, 3-methylbutanal, 3-methylbutanol, phenylacetaldehyde, and *trans*-furan linalool oxide dominate the aroma of concentrated blackberry juice (*28*, *34*).

Blackberry research has switched to develop thornless cultivars with attributes of high yield, machine-harvestable, cold tolerance and superior flavor.

ome thornless blackberries with very good flavor such as ‘Black Diamond’, ‘Black Pearl’ and ‘Nightfall’ have been recently released to the industry.

Blueberry

Blueberries are in the genus *Vaccinium*, section *Cyanococcus*. The species from which the cultivated types were developed are native only to North America. The genus consists of 24 blueberry species, including 7 diploid, 14 tetraploid, and 3 hexaploid species (*35*). Three predominant types of blueberry are cultivated commercially including the tetraploid highbush (*Vaccinium corymbosum*) and lowbush (*V. angustifolium*) blueberries, and the hexaploid rabbiteye (*V. virgatum*; formerly *V. ashei*) blueberries. Highbush blueberries are native to much of the eastern and northeastern U.S., from the Appalachian Mountains to the Atlantic Ocean; Lowbush blueberries are native from Minnesota to Virginia and to the northeastern U.S. and the Maritime Provinces of Canada; ‘Rabbiteye’ blueberries are native to the southeastern U.S.

Compared to raspberries, the blueberry flavor research is limited. Overall, fewer volatile compounds have been identified in blueberries than in raspberries and blackberries (Table 3). The flavor profiles in these species are similar and are dominated by terpenoids, C6 alcohols, and esters.

Table 3. Compounds identified in highbush, lowbush, Rabbiteye, and wild diploid blueberries

Compounds	*Highbush*	*Lowbush*	*Rabbiteye*	*Wild Diploid*
Esters				
Methyl acetate		(*40*)		(*35*)
Ethyl acetate	(*36*)	(*40, 41*)	(*42*)	(*35*)
Ethyl propanoate		(*41*)		(*35*)
Methyl 2-methylpropanoate		(*41*)		
Ethyl 2-methylpropanoate		(*41*)		(*35*)
Methyl butyrate		(*41*)		(*35*)
Methyl 2-methylbutanoate		(*4*)		
Methyl 3-methylbutanoate		(*4, 41*)		(*35*)
Ethyl butyrate		(*4, 41*)		(*35*)
Methyl 3-methyl-2-butenoate		(*41*)		
Ethyl 2 / 3-methylbutanoate		(*4, 41*)		(*35*)
Ethyl 3-methyl-2-butenoate		(*41*)		(*35*)

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Table 3. (Continued). Compounds identified in highbush, lowbush, Rabbiteye, and wild diploid blueberries

Compounds	*Highbush*	*Lowbush*	*Rabbiteye*	*Wild Diploid*
Ethyl hexanoate				(*35*)
Hexyl acetate	(*38*)			
Farnesyl acetate	(*38*, *39*)			
Geranyl formate			(*42*, *43*)	
Linalool acetate			(*42*)	
Terpinyl acetate			(*43*)	
Terpenoids				
α-Pinene	(*38*)			(*35*)
β-Pinene				(*35*)
Myrcene	(*38*)		(*43*)	(*35*)
Limonene	(*36*, *38*)	(*41*)	(*42*, *43*)	(*35*)
Eucalyptol	(*38*)		(*43*)	(*35*)
α-Terpinolene			(*43*)	(*35*)
Caryophyllene	(*38*)			
Allocimene			(*43*)	
Linalool	(*4*, *36*–*39*)	(*4*)	(*42*, *43*)	(*35*)
4-Terpieneol			(*42*, *43*)	
α-Terpineol	(*4*, *36*, *38*, *39*)		(*42*, *43*)	
Citronellol	(*38*, *39*)			
Nerol	(*36*, *38*, *39*)		(*42*, *43*)	
Geraniol	(*36*, *38*, *39*)		(*42*, *43*)	
Pulegone			(*43*)	
Carveol			(*42*)	
Hydroxycitronellol	(*38*, *39*)			
cis-Caren-3-ol			(*42*, *43*)	
Farnesol	(*38*, *39*)			
Cineralone			(*42*)	
Carbonyl compounds				

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Table 3. (Continued). Compounds identified in highbush, lowbush, Rabbiteye, and wild diploid blueberries

Compounds	*Highbush*	*Lowbush*	*Rabbiteye*	*Wild Diploid*
2 / 3-Methylbutanal				(*35*)
Hexanal	(*36*, *38*)		(*42*, *43*)	(*35*)
trans-2-Hexenal	(*36*, *38*, *39*)		(*42*, *43*)	(*35*)
Decanal				(*35*)
Diacetyl				(*35*)
2-Pentanone			(*42*, *43*)	
2-Heptanone		(*41*)		
2-Nonanone		(*41*)		(*35*)
2-Undecanone			(*42*)	
Norisoprenoids				
β-Ionone			(*42*, *43*)	
Alcohols				
3-Methylbutanol				(*35*)
1-Penten-3-ol	(*36*, *38*)		(*42*, *43*)	
1-Hexanol	(*36*, *38*, *39*)		(*42*, *43*)	(*35*)
cis-3-Hexenol	(*36–39*)		(*43*)	
trans-2-Hexenol	(*36*, *38*, *39*)		(*43*)	
Heptanol	(*36*)		(*42*)	
1-Octanol	(*38*)			
1-Nonanol	(*36*, *38*)			
Aromatic compounds				
p-Cymene		(*41*)	(*42*, *43*)	(*35*)
Benzyl alcohol	(*38*, *39*)	(*41*)	(*42*)	
Phenylethyl alcohol	(*38*, *39*)			
Cinnamyl alcohol	(*38*, *39*)			
Phenol	(*38*, *39*)			
Thymol	(*38*)		(*42*, *43*)	

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Table 3. (Continued). Compounds identified in highbush, lowbush, Rabbiteye, and wild diploid blueberries

Compounds	*Highbush*	*Lowbush*	*Rabbiteye*	*Wild Diploid*
Carvacrol			(*43*)	
Eugenol	(*38*, *39*)		(*42*, *43*)	
Isoeugenol	(*38*)			
Benzaldehyde	(*4*, *38*, *39*)	(*41*)	(*43*)	
Cinnamaldehyde	(*38*)			
Vanillin	(*38*, *39*)			
Acids				
Acetic acid	(*38*)			
Butanoic acid	(*38*)			
2-Methylbutanoic acid	(*38*)			
Pentanoic acid	(*38*)			
Hexanoic acid	(*38*)			
Others				
γ-Butyrolactone	(*4*)			
Dimethyl sulfide				(*35*)
6-ethyl-2, 6-decadiene-4, 5-diol	(*4*)			
2-furfural			(*42*, *43*)	
5-methylfurfural			(*42*, *43*)	
acetylfuran				

Highbush cultivars have large berries and pleasant aromas. Parliment and Kolor (*36*) first reported the major volatile compounds in highbush blueberries were ethyl acetate, *trans*-2-hexenal, *trans*-2-hexenol, hexanal, *cis*-3-hexenol, linalool, and geraniol. An aroma recombination study demonstrates that the combination of *trans*-2-hexenal, *trans*-2-hexenol, *cis*-3-hexenol and linalool gives the characteristic blueberry flavor (*36*, *37*). However, more recent studies (*4*, *38*, *39*) show that there are more compounds contributing to the aroma of highbush blueberries. GC-olfactometry and OAV studies suggest that *trans*-2-hexenal, geraniol, *cis*-3-hexen-1-ol, linalool, citronellol, a-terpineol, 2-phenylethanol and vanillin may contribute to the typical aroma of blueberries.

Only a few studies reported the aroma composition of lowbush blueberries (*4*, *40*, *41*). These studies identified the aroma-active compounds in lowbush blueberry as acetaldehyde, methyl acetate, ethyl acetate, methyl 2-methylbutanoate, methyl 3-methylbutanoate, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate, methyl butanoate and linalool. Apparently, more esters were reported in lowbush than in highbush blueberries.

Studies on the flavor of rabbiteye blueberries are also limited, and the literature comes from only one group. Horvat et al.(*42*, *43*) studied the volatile composition of rabbiteye blueberries using distillation, followed with pentane extraction, and gas chromatographic and mass spectrometric identification. The major components identified are ethyl acetate, *p*-cymene, hexanol, *cis*-2-hexenol, heptanol, cinerolone, β-ionone, terpene-4-ol, 2-undecanone, α-terpineol, carveol, nerol, and eugenol. A mixture of *cis*-3-hexenol, *trans*-2-hexenol, *trans*-2-hexenal, linalool, and geraniol produced a typical fruity aroma reminiscent of fresh rabbiteye blueberry flavor, as determined by informal sensory evaluations (*43*).

Little research has examined the effect of environmental factors on the volatile composition of blueberries. A comparison of the volatile constituents in rabbiteye (*43*, *44*) revealed that during ripening, concentrations of low molecular weight volatiles tend to decrease while higher molecular weight compounds increase. The compounds *trans*-2-hexenal, *trans*-2-hexenol, *cis*-3-hexenol, α-terpienol, and β-caryophyllene all decreased in concentration as the fruit progressed from green to midripe to fully ripe. However, linalool and geraniol concentrations were equal or greater in midripe and ripe fruit, than in green fruit.

Wild diploid blueberries have also been studied (*35*). Acetaldehyde, dimethyl sulfide, diacetyl, ethyl acetate, hexanal, ethyl 2-methylbutanoate, *trans*-2-hexenal, eucalyptol, and linalool have been identified.

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Conclusion and Perspective

A large number of volatile aroma compounds have been identified in raspberries, blackberries, and blueberries through qualitative, quantitative, and sensory evaluation. The investigation in raspberry flavor has been relatively extensive, while studies are still very limited for blackberry and blueberry flavor. Volatile aroma composition varies among cultivars and is affected by environmental factors such as geographical location, soil nutrition, weather, and irrigation.

A better understanding of the volatile chemicals responsible for berry flavor is needed. New methodologies must be developed to further identify and quantify aroma compounds in fresh berry crops. For instance, some polar aroma compounds have berry flavor, but are hard to analyze with common methods. Efforts need to continue combining sensory evaluation and chemical analysis to develop a complete picture of the chemical basis of aroma and flavor.

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References

1. Honkanen, E.; Hirvi, T. The flavor of berries. *Dev. Food Sci.* **1990**, *3C*, 125–193, Food Flavours, Pt. C.
2. Seeram, N. P. Berry fruits for cancer prevention: Current status and future prospects. *J. Agric. Food Chem.* **2008**, *56* (3), 630–635.
3. Seeram, N. P.; Adams, L. S.; Zhang, Y.; Lee, R.; Sand, D.; Scheuller, H. S.; Heber, D. Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. *J. Agric. Food Chem.* **2006**, *54* (25), 9329–9339.
4. Forney, C. F. Horticultural and other factors affecting aroma volatile composition of small fruit. *HortTechnology* **2001**, *11* (4), 529–538.
5. Honkanen, E. P.; Tapani; Hirvi, T. The aroma of Finnish wild raspberries, *Rubus idaeus*, L. *Z. Lebensm. Unters. Forsch.* **1980**, *171* (3), 180–182.
6. Pyysalo, T. Identification of volatile compounds in hybrids between raspberry (*Rubus idaeus*, L.) and arctic bramble (*Rubus arcticus*, L.). *Z. Lebensm. Unters. Forsch.* **1976**, *162* (3), 263–272.
7. Larsen, M. P.; Poll, L.; Callesen, O.; Lewis, M. Relations between the content of aroma compounds and the sensory evaluation of 10 raspberry varieties (*Rubus idaeus*, L.). *Acta Agric. Scand.* **1991**, *41*, 447–454.
8. Shamaila, M.; Skura, B.; Daubeny, H.; Anderson, A. Sensory, chemical, and gas chromatographic evaluation of five raspberry cultivars. *Food Res. Inter.* **1993**, *26*, 443–449.
9. Wustenfeld, H.; Luckow, C. Evaluation of raspberry juices. *Z. Unters. Lebensm.* **1931**, *61*, 341–345.
10. Klesk, K.; Qian, M.; Martin, R. R. Aroma extract dilution analysis of cv. Meeker (*Rubus idaeus*, L.) red raspberries from Oregon and Washington. *J. Agric. Food Chem.* **2004**, *52* (16), 5155–5161.
11. Vereshchagin, P. V.; Bezzubov, A. A. Study of the composition of raspberry essential oils. *Prikladnaya Biokhimiya i Mikrobiologiya* **1981**, *17* (1), 67–72.
12. Renner, R.; Hartmann, U. Aroma substances in raspberry essence and their evaluation. *Lebensmittelchem. Gerichtl. Chem.* **1985**, *39*, 30–32.
13. Roberts, D. D.; Acree, T. E. Effects of heating and cream addition on fresh raspberry aroma using a retronasal aroma simulator and gas chromatography olfactometry. *J. Agric. Food Chem.* **1996**, *44* (12), 3919–3925.
14. Larsen, M.; Poll, L. Odor thresholds of some important aroma compounds in raspberries. *Z. Lebensm. Unters. Forsch.* **1990**, *191* (2), 129–131.
15. Borejsza-Wysocki, W.; Goers, S. K.; McArdle, R. N.; Hrazdina, G. (p-Hydroxyphenyl)butan-2-one levels in raspberries determined by chromatographic and organoleptic methods. *J. Agric. Food Chem.* **1992**, *40* (7), 1176–1177.
16. Schinz, H.; Seidel, C. F. Aromatic materials. I. Raspberry aromatic materials. *Helv. Chimica Acta* **1957**, *40*, 1839–1859.
17. Braun, G.; Hieke, E. The analysis of aromatic substances in foods. Part III. Information on the content of 1-(4’-hydroxyphenyl)butan-3-one (raspberry ketone) in raspberries and raspberry foods and the determination

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of artificial flavoring with 1-(4'-hydroxyphenyl)butan-3-one. *Deutsche Lebensmittel-Rundschau* **1977**, *73* (9), 273–278.

18. Gallois, A. Rapid determination of 1-(4-hydroxyphenyl)-3-butanone in raspberries by thin-layer chromatography. *Sci. Aliments* **1982**, *2* (1), 99–106.
19. Maquin, F.; Meili, M.; Chaveron, H. Determination of 4-(p-hydroxyphenyl)-2-butanone by mass fragmentometry. *Annales des Falsifications de l'Expertise Chimique et Toxicologique* **1981**, *74* (800), 511–521.
20. Moore, P. P. Genotype x environment variation in raspberry fruit aroma volatiles. *Acta Hortic.* **2002**, *585* (2), 511–516.
21. Malowicki, S. M. M.; Martin, R.; Qian, M. C. Volatile composition in raspberry cultivars grown in the Pacific Northwest determined by stir bar sorptive extraction−gas chromatography−mass spectrometry. *J. Agric. Food Chem.* **2008**, *56* (11), 4128–4133.
22. Guichard, E. Comparison of two nitrogen entrainment methods for the extraction of volatile constituents from raspberry. *Sci. Aliments* **1984**, *4* (2), 317–324.
23. Robertson, G. W.; Griffiths, D. W.; Woodford, J. A. T.; Birch, A. N. E. Changes in the chemical composition of volatiles released by the flowers and fruits of the red raspberry (*Rubus idaeus*) cultivar Glen Prosen. *Phytochemistry* **1995**, *38* (5), 1175–1179.
24. De Ancos, B.; Ibanez, E.; Reglero, G.; Cano, M. P. Frozen storage effects on anthocyanins and volatile compounds of raspberry fruit. *J. Agric. Food Chem.* **2000**, *48* (3), 873–879.
25. Scanlan, R. A.; Bills, D. D.; Libbey, L. M. Blackberry flavor components of commercial essence. *J. Agric. Food Chem.* **1970**, *18* (4), 744.
26. Gulan, M. P.; Veek, M. H.; Scanlan, R. A.; Libbey, L. M. Compounds identified in commercial blackberry essence. *J. Agric. Food Chem.* **1973**, *21* (4), 741.
27. Georgilopoulos, D. N.; Gallois, A. N. Aroma compounds of fresh blackberries (*Rubus laciniata*, L.). *Z. Lebensm. Unters. Forsch.* **1987**, *184* (5), 374–380.
28. Georgilopoulos, D. N.; Gallois, A. N. Flavor compounds of a commercial concentrated blackberry juice. *Food Chem.* **1988**, *28* (2), 141–148.
29. Klesk, K.; Qian, M. Preliminary aroma comparison of Marion (*Rubus* spp. *hyb*) and Evergreen (*R. laciniatus*, L.) blackberries by dynamic headspace/ OSME technique. *J. Food Sci.* **2003**, *68* (2), 697–700.
30. Klesk, K.; Qian, M. Aroma extract dilution analysis of cv. Marion (*Rubus* spp. *hyb*) and cv. Evergreen (*R. laciniatus*, L.) blackberries. *J. Agric. Food Chem.* **2003**, *51* (11), 3436–3441.
31. Qian, M. C.; Wang, Y. Seasonal variation of volatile composition and odor activity value of Marion (*Rubus* spp. *hyb*) and Thornless Evergreen (*R. laciniatus*, L.) blackberries. *J. Food Sci.* **2005**, *70* (1), C13–C20.
32. Du, X.; Qian, M. Quantification of 2,5-dimethyl-4-hydroxy-3(2H)-furanone using solid-phase extraction and direct microvial insert thermal desorption gas chromatography−mass spectrometry. *J. Chromatogr., A* **2008**, *1208*, 197–201.

33. Wang, Y.; Finn, C.; Qian, M. C. Impact of growing environment on Chickasaw blackberry (*Rubus*, L.) aroma evaluated by gas chromatography olfactometry dilution analysis. *J. Agric. Food Chem.* **2005**, *53* (9), 3563–3571.
34. Georgilopoulos, D. N.; Gallois, A. N. Volatile flavor compounds in heated blackberry juices. *Z. Lebensm. Unters. Forsch.* **1987**, *185* (4), 299–306.
35. Baloga, D. W.; Vorsa, N.; Lawter, L. Dynamic headspace gas chromatography−mass spectrometry analysis of volatile flavor compounds from wild diploid blueberry species. *ACS Symposium Series* **1995**, *596*, 235–247, Fruit Flavors.
36. Parliment, T. H.; Kolor, M. G. Identification of the major volatile components of blueberry. *J. Food Sci.* **1975**, *40* (4), 762–763.
37. Parliment, T. H.; Scarpellino, R. Organoleptic techniques in chromatographic food flavor analysis. *J. Agric. Food Chem.* **1977**, *25* (1), 97–99.
38. Hirvi, T.; Honkanen, E. The aroma of blueberries. *J. Sci. Food Agric.* **1983**, *34* (9), 992–996.
39. Hirvi, T.; Honkanen, E. The aroma of some hybrids between high-bush blueberry (*Vaccinium corymbosum*, L.) and bog blueberry (*Vaccinium uliginosum*, L.). *Zeitschrift fuer Lebensmittel-Untersuchung und -Forschung* **1983**, *176* (5), 346–349.
40. Hall, I. V.; Frsyth, F. R.; Lightfoot, H. J. Volatiles from developing fruit of *Vaccinium angustifolium*. *Can. Inst. Food Technol. J.* **1970**, *3* (1), 1–3.
41. Lugemwa, F. N.; Lwande, W.; Bentley, M. D.; Mendel, M. J.; Alford, A. R. Volatiles of wild blueberry, *Vaccinium angustifolium*: Possible attractants for the blueberry maggot fruit fly, *Rhagoletis mendax*. *J. Agric. Food Chem.* **1989**, *37* (1), 232–233.
42. Horvat, R. J.; Senter, S. D.; Dekazos, E. D. GLC-MS analysis of volatile constituents in rabbiteye blueberries. *J. Food Sci.* **1983**, *48* (1), 278–279.
43. Horvat, R. J.; Senter, S. D. Comparison of the volatile constituents from rabbiteye blueberries (*Vaccinium ashei*) during ripening. *J. Food Sci.* **1985**, *50* (2), 429–431.
44. Horvat, R. J.; Schlotzhauer, W. S.; Chortyk, O. T.; Nottingham, S. F.; Payne, J. A. Comparison of volatile compounds from rabbiteye blueberry (*Vaccinium ashei*) and deerberry (*V. stamineum*) during maturation. *J. Essent. Oil Res.* **1996**, *8* (6), 645–648.
45. Du, X.; Qian, M. Quantification of 2,5-dimethyl-4-hydroxy-3(2H)-furanone using solid-phase extraction and direct microvial insert thermal desorption gas chromatography−mass spectrometry. *J. Chromatogr., A* **2008**, *1208* (1-2), 197–201.

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Chapter 4

Fractionation and Identification of Aroma-Active Constituents in Thornless Trailing 'Black Diamond' Blackberry

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The aroma constituents of thornless 'Black Diamond' blackberry were isolated by dichloromethane liquid-liquid extraction followed by solvent-assisted flavor evaporation. The volatile extract was fractionated to an acidic fraction and a neutral fraction; the neutral fraction was further chromatographed on a packed silica gel column. Pentane, pentane-diethyl ether (95:5, 90:10, 50:50), and methanol were used to fractionate the volatile compounds. The pentane fraction mainly consisted of hydrocarbons, esters were in the 95:5 pentane-diethyl ester fraction, aldhydes and ketones were in the 90:10 pentane-diethyl esther fraction, while alcohols and lactones were in the 50:50 pentane-diethyl ether fraction. Methanol fraction contained hydroxyl esters and furanones. The aroma-active compounds in each fraction as well as the mixture were identified using gas chromatography-olfactometry-mass spectrometry. The results indicated the most potentially important odor-active compounds in 'Black Diamond' was ethyl butanoate, ethyl hexanoate, 1-octen-3-one, 2-heptanol, *cis*-3-hexenol, nonanal, *trans*-2-hexenol, methional, linalool, ethyl 3-hydroxyhexanoate, α-ionone, β-ionone, furaneol, and 5-isoprenyl-2-dimethyl-divinyltetrahydrofuran.

In Flavor and Health Benefits of Small Fruits; Qian, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

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Introduction

'Black Diamond' was a recently released thornless blackberry in the Pacific Northwest of America (*1*). The outstanding characteristics of 'Black Diamond' include large, uniformly shaped and firm fruit, high yield, good processed fruit quality, excellent adaptation to machine harvesting, and good disease and winter injury tolerance (*1*). Since its commercial release, 'Black Diamond' has been the most commonly planted cultivar, with 55% more plants planted than 'Marion' (P. Moore, pers. comm.). As fruit of 'Black Diamond' become a more important constituent of the processed blackberry supply, understanding its aroma profile will be ciritical. However, no study has been focused on the aroma profile of 'Black Diamond'.

Blackberry flavor is a rather complex mixture of volatiles with different functionalities (*2–9*). The diverse volatile components include esters, aldehydes, ketones, lactones, terpenoids, norisoprenoids, alcohols, phenolics, furanones, and acids. Volatile compounds such as 2-heptanol, *p*-cymen-8-ol, 2-heptanone, 1-hexanol, α-terpineol, pulegone, 1-octanol, isoborneol, myrtenol, 4-terpineol, carvone, elemicine, and nonanal have been identified as the major volatiles in 'Evergreen' blackberry. Using gas chromatography-olfactometry, 2-methylbutanal, 3-methylbutanal, ethyl 2-methylbutanoate, ethyl hexanoate, hexanal, *trans*-2-hexenal, 2-heptanol, ß-ionone, linalool, 2-heptanone, 2-undecanone, α-ionone, 2,5-dimethyl-4-hydroxy-3-(2*H*)-furanone, 2-ethyl-4-hydroxy-5-methyl-3-(2*H*)-furanone, 4-hydroxy-5-methyl-3-(2*H*)-furanoen, 4,5-dimethyl-3-hydroxy-2-(5*H*)-furanone, and 5-ethyl-3-hydroxy-4-methyl-2-(5*H*)-furanone, dimethyl trisulfide, and methional have been identified as the major aroma compounds in 'Thornless Evergreen' and 'Marion' blackberry (*7*, *8*).

It is hard to analyze these diverse and complex volatile compounds by a single gas chromatograph. Preseparation of flavor compounds prior to GC analysis, where the chemical compounds of a complex sample are separated into classes based on their chemical or physical properties, has proven to be a helpful strategy (*10*). Pre-separation with silica gel normal phase chromatography has been used to help the identification of aroma compounds in 'Chichasaw' blackberry (*11*). Fractionation based on the functional group and polarity on silica gel simplifies the composition of each fraction and facilitates the aroma-active compound identification during GC-olfactometry analysis (*11*).

The objective of this study is to identify aroma profile of the newly released thornless 'Black Diamond' blackberry. The better understanding of the flavor profile and chemical composition can be used to guide the breeding program to further develop high quality thornless blackberry cultivars.

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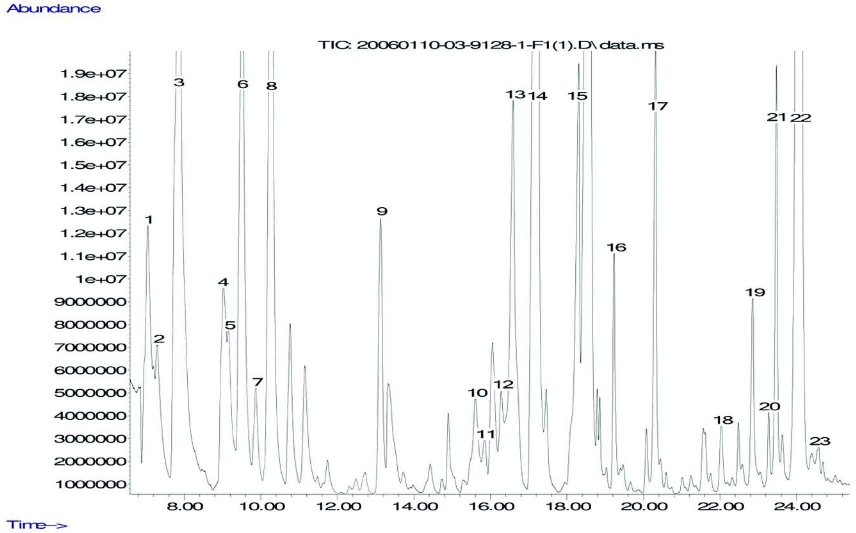

Figure 1. Chromatogram of fraction 1 (pentane) obtained by normal-phase chromatography of neutral fraction of Black Diamond blackberry extract. Key: (1) myrcene; (2) α-terpinene; (3) limonene; (4) cis-β-ocimene; (5) γ-terpinene; (6) trans-β-ocimene; (7) p-cymene; (8) α-terpinolene; (9) allo-ocimene; (10) α-cubebene; (11) δ-elemene; (12) α-ylangene; (13) α-copaene; (14) δ-carene; (15) isoledene; (16) megstigma-4,6(E),8(E)-triene; (17) megstigma-4,6(Z),8(Z)-triene; (18) α-gurjunene; (19) α-longipinene; (20) azulene; (21) 1,1,6-trimethyl-1,2-dihydronaphthalene; (22) α-farnesene; (23) δ-cadinene.

Experimental

Chemicals

Calcium chloride, sodium chloride and anhydrous sodium sulfide were obtained from Fisher Scientific (Fair Lawn, NJ). Solvents of dichloromethane (Burdick & Jackson, Muskegon, MI), pentane (Mallinckrodt Baker, Phillipsburg, NJ), and diethyl ether were analytical grade and purified by distillation. Methanol (HPLC grade) was from EM Science (Gibbstown, NJ).

6,6-Dimethyl-5-methylidenebicyclo[2.2.1]heptane (camphene), 1-methyl-4-prop-1-en-2-ylcyclohexene (limonene), 1-methyl-4-propan-2-ylbenzene (*p*-cymene), 1-methyl-4-propan-2-ylidenecyclohexene (α-terpinolene), ethyl butanoate, methyl hexanoate, ethyl hexanoate, hexyl acetate, heptyl acetate, hexanal, *trans*-2-hexenal, octan-1-al, 1-octen-3-one, nonan-1-al, *trans*-2-nonanal, *trans*, *cis*-2, 6-nonadienal, cinnamaldehyde, (3*E*)-4-(2,6,6-trimethylcyclohex-1-en-1-yl)but-3-en-2-one (β-ionone), 2-heptanol, *cis*-2-hexenol, heptanol, 3,7-dimethylocta-1,6-dien-3-ol (linalool), 3,7-dimethyloct-6-en-1-ol (citronellol), (2*Z*)-3,7-dimethylocta-2,6-dien-1-ol (nerol), 3,7-dimethylocta-2,6-dien-1-ol (geraniol), 4-(2,6,6-trimethyl-1-cyclohex-2-enyl)but-3-en-2-ol (α-ionol), 2,6,6,10-tetramethyl-1-oxaspiro[4.5]dec-9-ene (theaspirane), 4-methoxy-2,5-dimethyl-3(2*H*)-furanone (mesifurane), 4-decanolide-5-hexyldihydro-2(3*H*)-furanone (γ-decalactone), 3-hydroxy-4,5-dimethylfuran-2(5*H*)-one (sotolon), acetic acid, butanoic acid, 2-methylbutanoic acid, hexanoic acid, and octanoic acid were obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI). 2-Heptanone, hexanol, benzyl alcohol, and phenylethanol were obtained from Sigma (Milwaukee, WI). 3,7-Dimethylocta-1,3,6-triene (ocimene), (3*E*)-4-(2,6,6-Trimethylcyclohex-2-en-1-yl)but-3-en-2-one (α-ionone), 4-hydroxy-3-methoxybenzaldehyde (vanillin), 6-methyl-2-(oxiran-2-yl)hept-5-en-2-ol (linalool oxide), 4-hydroxy-2,5-dimethyl-3-furanone (furaneol), and pentanoic acid were obtained from Fluke (Buchs, Switzerland). 7-Methyl-3-methylideneocta-1,6-diene (myrcene), isoamyl hexanoate, octyl acetate, undecan-2-one, nonan-2-ol, and 2-(4-Methyl- 1-cyclohex-3-enyl) propan- 2-ol (α-terpineol) was obtained from K & K Laboratories (Jamaica, NY). 1-Methyl-4-propan-2-ylcyclohexa-1,3-diene (α-terpinene), benzenepropanol, and cinnamyl alcohol were obtained from TCI Japan (Tokyo, Japan). *trans*-3-Hexenol and *cis*-3-hexenol were obtained from Bedoukian Research (Danbury, CT). *cis*-4-Heptenal, *trans*-2-octenal, and *trans*-2-Hexenol, were obtained from Compagnie Parento, Inc (Lenior, NC). (*E*)-1-(2,6,6-Trimethyl-1-cyclohexa-1,3-dienyl)but-2-en-1-one (β-damascenone) was obtained from Firmenich (Princeton, NJ). Methyl cinnamate, acetophenone, and 4-octanolide-4,5-dihydro-5-butyl-2(3*H*)-furanone (γ-octalactone) were from Alfa Aesar (Ward Hill, MA). Ethyl octanoate, octan-1-ol, and nonan-1-ol were from Eastman Blue (Rochester, NY).

Blackberry Samples

Full ripe 'Black Diamond' fruit (°Brix 10.5±0.5, titratable acidity 1.1±0.1%, shiny black) were hand harvested from plants growing in research plots at Oregon State University Lewis-Brown Farm in Corvallis, Oregon, between June and July of 2004 growing seasons. The fruits were individually quick frozen (IQF) immediately after harvest and stored at -18 °C until analysis. The frozen fruits were thawed in a refrigerator (1 °C) overnight. After addition of 1% of calcium chloride and 20% sodium chloride (final concentration), the sample was blended in a glass blender (Waring Products Div., Dynamics Corp. of America, New Hartford, CT) to a fine puree.

Volatile Compound Extraction

Five hundred grams of fresh puree were transferred to a 2 L Erlenmeyer flask covered with aluminum foil and extracted with 200 mL of freshly distilled dichloromethane for 12 h on a platform shaker (Innova 2300; New Brunswick Scientific, Edison, NJ) at 130 rpm. The solvent and puree mixture was centrifuged

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at 2000 rpm for 15 min. The organic phase was collected and the juice and the puree were returned to flask for further extraction. The procedure was repeated twice with 150 and 100 mL of dichloromethane each. All organic extracts were combined. Volatile compounds were recovered by using solvent-assisted flavor evaporation (SAFE) at 50 °C under a vacuum of 2 Bar. The organic SAFE distillate was dried with anhydrous sodium sulfate. Then it was concentrated to ~30 mL by solvent distillation at 30 °C under a vacuum of 460 Bar, followed by concentration to ~2 mL in a micro-Kuderna-Danish concentrator and then to ~500 μL with a gentle stream of nitrogen. The extract was labeled as "mixture".

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Volatile Fractionation

Using the same procedure, another 30 mL of concentrated SAFE distillate was prepared for fractionation. The concentrated was mixed with 15 mL of sodium bicarbonate solution (pH 9.0), saturated with sodium chloride, and then separated in a separatory funnel. The aqueous phase was saved and the organic phase was extracted two more times with 10 and 5 mL of sodium bicarbonate solution each time.

All the aqueous phases were mixed, and the pH was adjusted to 2.0 with 2 N sulfuric acid, and then the solution was extracted with diethyl ether for three times (15 + 10 + 5 mL). The diethyl ether extract was combined and dried with anhydrous sodium sulfate, and concentrated to ~500 μL under a gentle stream of nitrogen. This concentrate was labeled as "acidic fraction".

The dichloromethane solution separated from aqueous solution was dried over anhydrous sodium sulfate and concentrated to a final volume of 1 mL using the aforementioned procedure. It was labeled as "neutral fraction".

The neutral fraction was fractionated by normal phase chromatography on silica gel (EM Science, 35-70 mesh, 60 Å) described by Qian and Reineccius (*12*) with some modification. Ten grams of silica gel, which had been soaked in methanol for more than 24 h, was packed into a 1.5 cm i.d. × 30 cm glass column. The column was washed with 50 mL of methanol and diethyl ether respectively and equilibrated in pentane. After sample was loaded (1 mL, concentrated), fractions were recovered by 40 mL of each following solvent: pentane (fraction 1), pentane and diethyl ether (fraction 2, 95:5; fraction 3, 90:10; fraction 4, 50:50, v/v), and methanol (fraction 5) at a flow rate of ~ 1.0 mL/min. All fractions were concentrated to a final volume of 200 μL under a stream of nitrogen and labeled as "NF1", "NF2", "NF3", "NF4", and "NF5", respectively.

Gas Chromatography-Olfactometry-Mass Spectrometry Analysis

GC-MS analyses were performed using an Agilent 6890 gas chromatograph equipped with an Agilent 5973 mass selective detector and an olfactometer. Chemical separation was achieved with a ZB-WAX column (30 m × 0.25 mm i.d. cross-linked polyethylene glycol, 0.25 μm film thickness, Phenomenex, Torrance, CA) (for all fractions and the mixture) and a DB-5 column (30 m × 0.25 mm i.d. cross-linked phenyl-methyl polysiloxane, 0.25 µm film thickness, J & W Scientific, Folsom, CA)(mixture only). The column effluent was split 1:1 (by volume) into the MS and a heated sniffing port with a fused silica outlet splitter (Alltech Associates, Inc., Deerfield, IL). A constant helium column flow was 2.5 mL/min, and the 2 µL sample was injected in the splitless mode. The oven temperature was programmed at 40 °C for a 2 min holding, then to 230 °C at 4 °C/min with 10 min holding. MS transfer line and ion source temperature were 280 and 230 °C, respectively. Electron impact mass spectrometric data from *m*/*z* 35~350 were collected using a scan model using a scan rate of 5.27/s, with an ionization voltage of 70 eV.

The olfactometry analyses of all samples including the "acidic fraction", "NF1", "NF2", "NF3", "NF4", and "NF5" and the "mixture" were achieved by three experienced panelists. The odor intensities were evaluated in a 15 point intensity scale, where 1 meant a volatile had a slightly sensory impact, 7 was for moderate, 11was for large, and 15 was for extreme impact. Duplicate analyses were performed for each sample by each panelist. The intensity was the average from all panelists when an aroma was registered.

Compound identifications were made by comparing mass spectral data from the Wiley 275.L (G1035) Database (Agilent) and confirmed by comparing Kovats retention index to standards or those reported in the literature, in addition to odor descriptions.

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Results and Discussion

'Black Diamond' Aroma Fractionation

The aroma extract of the 'Black Diamond' was very complex. To simplify the chromatogram, and facilitate compound identification and organoleptic recognition, a pre-fractionation of the aroma extract was performed prior to GC analysis. The extract was fractionated to an acidic fraction and a neutral fraction.

The acidic fraction had strong acidic notes, including enrichment in the most water soluble compounds such as short chain fatty acids (Table I). Acetic acid, butyric acid, and hexanoic acid had very strong organoleptic properties. However, this fraction did not possess a berry-like odor. The acidic fraction was relatively simple and positive identification for all aroma compounds was achieved.

Table I. Odor-Active Compounds Identified in Fractions of 'Black Diamond'

Compounds	*RI (Wax)*[a]	*Fr.*[b]	*Descriptors*	*Identi.*[c] *basis*
camphene	1069	1	camphor	MS, RI, aroma
sabinene	1112	1	fatty, woody	MS, RI, aroma
β-myrcene	1180	1	spicy, musty	MS, RI, aroma

Continued on next page.

Table I. (Continued). Odor-Active Compounds Identified in Fractions of 'Black Diamond'

Compounds	*RI (Wax)*[a]	*Fr.*[b]	*Descriptors*	*Identi.*[c] *basis*
δ-3-carene*	1190	1	gas, sweet	MS
limonene	1213	1	citrus	MS, RI, aroma
cis-β-ocimene	1259	1	citrus, herb	MS, RI, aroma
trans-β-ocimene	1278	1	citrus	MS, RI, aroma
p-cymene	1292	1	lemon, sweet, spicy	MS, RI, aroma
α-terpinolene	1309	1	woody, camphor	MS, RI, aroma
γ-cadinene*	1566	1	citrus, sweet	MS
ethyl butanoate	1047	2	fruity, flower	MS, RI, aroma
methyl hexanoate	1207	2	fruity, floral	MS, RI, aroma
ethyl hexanoate	1266	2	fruity, green	MS, RI, aroma
hexyl acetate	1304	2	green	MS, RI, aroma
heptyl acetate	1425	2	fatty, green	MS, RI, aroma
ethyl octanoate	1501	2	peanut	MS, RI, aroma
isoamyl hexanoate	1527	2	fruity, green	MS, RI, aroma
octyl acetate	1551	2	green, earthy, herbal	MS, RI, aroma
theaspirane A	1571	2	green, earthy, floral	MS, RI, aroma
theaspirane B	1623	2	green tea, floral	MS, RI, aroma
hexanal	1076	3	fruity,green	MS, RI, aroma
2-heptanone	1195	3	banana, fruity	MS, RI, aroma
trans-2-hexenal	1237	3	green, leaf	MS, RI, aroma
cis-4-heptenal	1265	3	fatty,musty	MS, RI, aroma
octanal	1320	3	green, fruity, fatty	MS, RI, aroma
1-octen-3-one	1333	3	musty, earthy, green	MS, RI, aroma
trans-2-heptenal	1356	3	pungent, grassy, fatty	MS, RI, aroma
cis-rose oxide	1391	3	rose, floral	MS, RIL, aroma
trans-rose oxide	1406	3	green, rose	MS, RIL, aroma
nonanal	1448	3	fatty	MS, RI, aroma
trans-2-octenal	1484	3	green, leaf, spicy	MS, RI, aroma
dill ether*	1581	3	dill, mint	MS, aroma
benzaldehyde	1599	3	almond, woody	MS, RI, aroma

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Table I. (Continued). Odor-Active Compounds Identified in Fractions of 'Black Diamond'

Compounds	*RI (Wax)*[a]	*Fr.*[b]	*Descriptors*	*Identi.*[c] *basis*
cis-2-nonenal	1617	3	leaf, grassy	MS, RI, aroma
trans, *cis*-2, 6-nonadienal	1683	3	fresh, green, cucumber	MS, RI, aroma
2-undecanone	1708	3	citrus	MS, RI, aroma
dimethyltrisulfide	1765	3	rotten cabbage	MS, RI, aroma
acetophenone	1767	3	almond, leaf	MS, RI, aroma
trans-β-damascenone	1994	3	sweet, berry	MS, RI, aroma
citronellyl acetate	1795	3	green, fruity	MS, RI, aroma
dihydro-β-ionone	2012	3, 4	green, woody	MS, RI, aroma
α-ionone	2037	3	violet, berry	MS, RI, aroma
geranyl acetone	2051	3	fresh, fruity, rosy	MS, RI, aroma
β-ionone	2155	3	tea, berry	MS, RI, aroma
cinnamaldehyde	2244	3, 4	spicy, cinnamon	MS, RI, aroma
methyl cinnamate	2294	3	berry	MS, RI, aroma
2-heptanol	1355	4	moldy, musty	MS, RI, aroma
hexanol	1383	4	green, citrus	MS, RI, aroma
trans-3-hexenol	1389	4	green, moss	MS, RI, aroma
cis-3-hexenol	1413	4	leaf, green, grassy	MS, RI, aroma
trans-2-hexenol	1444/	4	green, leaf, rancid	MS, RI, aroma
cis-2-hexenol	1450	4	woody, green	MS, RI, aroma
cis-linalool oxide	1478	4	woody, floral,	MS, RI, aroma
heptanol	1507	4	leaf, fatty	MS, RI, aroma
trans-linalool oxide	1515	4	flower, green	MS, RI, aroma
2-nonanol	1587	4	fresh, green	MS, RI, aroma
linalool	1623	4	citrus, green, sweet	MS, RI, aroma
octanol	1643	4	mushroom	MS, RI, aroma
mesifurane	1666	4	caramel, strawberry	MS, RI, aroma
cis-6-nonenol	1704	4	fatty, green	MS, RI, aroma
nonanol	1779	4	citrus, green, tea	MS, RI, aroma

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Table I. (Continued). Odor-Active Compounds Identified in Fractions of 'Black Diamond'

Compounds	*RI (Wax)*[a]	*Fr.*[b]	*Descriptors*	*Identi.*[c] *basis*
α-terpineol	1823	4	green, fruity	MS, RI, aroma
trans-epoxylinalool*	1863	4	sweet, woody	MS
β-citronellol	1913	4	green, peach	MS, RI, aroma
myrtenol	1931	4	medicinal, woody	MS, RI, aroma
nerol	1952	4	green, tea	MS, RI, aroma
p-cymen-8-ol	2013	4	citrus, cooked berry	MS, RI, aroma
trans-geraniol	2025	4	citrus, fruity, berry	MS, RI, aroma
benzenemethanol	2046	4	berry, floral	MS, RI, aroma
α-ionol	2083	4	tea, berry	MS, RI, aroma
benzeneethanol	2090	4	rose, tea, berry	MS, RI, aroma
p-menth-1-en-9-ol*	2118	4	ginger, spicy	MS
dihydro-α-ionol*	2136	4	green tea	MS
dihydro-β-ionol*	2175	4	green tea	MS
γ-octalactone	2233	4	coconut, fruity	MS, RI, aroma
benzenepropanol	2262	4	berry, fruity	MS, RI, aroma
γ-decalactone	2389	4	fruity, berry	MS, RI, aroma
8-acetoxylinalool*	2515	4	tea, fruity	MS
cinnamyl alcohol	2594	4	sweet, tea, fatty	MS, RI, aroma
ethyl 3-hydroxybutanoate*	1600	5, A	floral, fruity	MS, RI
ethyl 4-hydroxybutanoate*	1952	5	fresh, tea	MS
methyl 5-hydroxyhexanoate*	2010	5	fresh, tea, sweet	MS
furaneol	2249	5, A	caramel, burnt sugar	MS, RI, aroma
vanillin	2974	5, A	creamy, vanilla	MS, RI, aroma
acetic acid	1447	A	vinegar, sour	MS, RI, aroma
2-methylpropanoic acid*	1636	A	sour	MS, aroma
butanoic acid	1691	A	sour, rancid, cheesy	MS, RI, aroma

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Table I. (Continued). Odor-Active Compounds Identified in Fractions of 'Black Diamond'

Compounds	*RI (Wax)*[a]	*Fr.*[b]	*Descriptors*	*Identi.*[c] *basis*
2-methylbutanoic acid	1773	A	sour, sweaty	MS, RI, aroma
pentanoic acid	1866	A	sour	MS, RI, aroma
4-pentenoic acid*	1960	A	sour	MS, aroma
2-pentenoic acid*	1966	A	sour, fruity	MS, aroma
hexanoic acid	2033	A	sour, pungent, rancid	MS, RI, aroma
octanoic acid	2304	A	sour	MS, RI, aroma
sotolon	2474	A	burnt, woody	MS, RI, aroma

[a] Retention index in ZB-WAX column; [b] Fractions from 1~5 and A was acid part; [c] Identification basis. RI: retention index from standard; RIL: retention index from the literature.

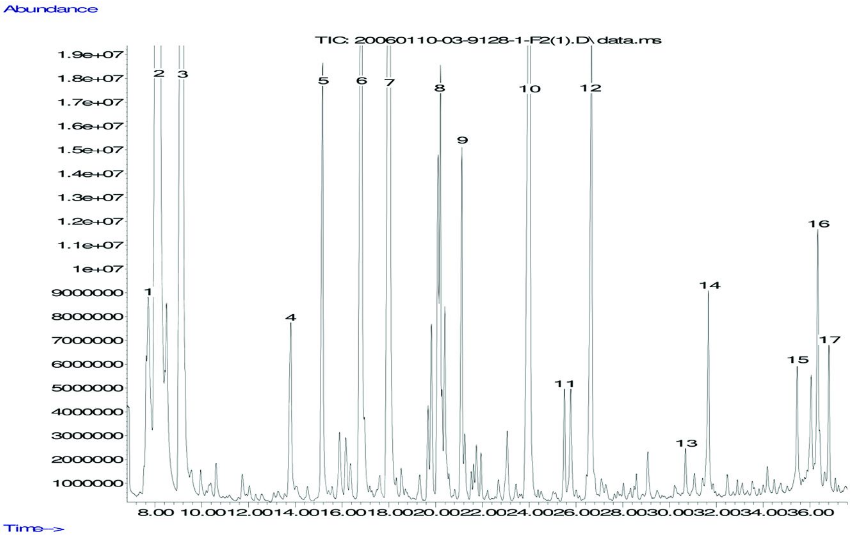

Figure 2. Chromatogram of fraction 2 (pentane:diethyl ether, 95:5) obtained by normal-phase chromatography of neutral fraction of Black Diamond extract. Key: (1) methyl hexanoate; (2) eucalyptol (IS); (3) ethyl hexanoate; (4) nonanal; (5) ethyl octanoate; (6) theaspirane A; (7) theaspirane B; (8) 1-p-menthen-9-al; (9) ethyl decanoate; (10) ethyl undecanoate; (11) methyl dodecanoate; (12) ethyl dodecanoate; (13) methyl tetradecanoate; (14) ethyl tetradecanoate; (15) methyl hexadecanoate; (16) ethyl hexadecanoate; (17) ethyl 9-hexadecanoate.

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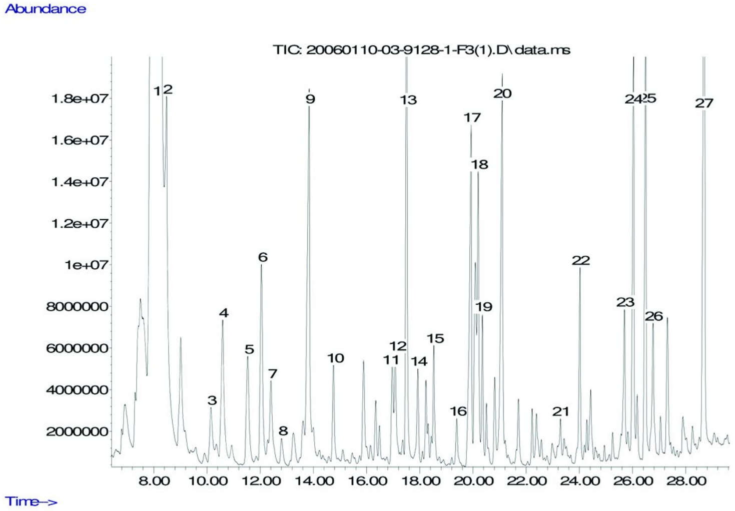

Figure 3. Chromatogram of fraction 3 (pentane:diethyl ether, 90:10) obtained by normal-phase chromatography of neutral fraction of Black Diamond extract. Key: (1) eucalyptol (IS); (2) trans-2-hexenal; (3) hexyl acetate; (4) octanal; (5) trans-2-heptenal; (6) trans-2-hexyl acetate; (7) trans-rose oxide; (8) cis-rose oxide; (9) nonanal; (10) trans-2-octenal; (11) decanal; (12) dill ether; (13) benzaldehyde; (14) trans-2-nonenal; (15) linalool; (16) trans-2-cis-6-nonadienal; (17) 2-undecanone; (18) 1-p-menthen-9-al; (19) myrtenal; (20) trans-2-decenal; (21) carvone; (22) undecanal; (23) trans-β-damascenone; (24) dihydro-β-ionone; (25) α-ionone; (26) geranyl acetone; (27) β-ionone.

The neutral fraction was complex and reminiscent of blackberry odor, it was further separated on a silica gel column to five fractions. An excellent separation of aroma compounds by polarity was achieved (Figures 1−5).

The first pentane fraction was almost odorless with a slight citrus aroma. This fraction contained only hydrocarbon compounds (Table I). Although many fruits often contain high amounts of hydrocarbons, this class does not generally contribute to the aroma of the fruit. However, the pentane fraction can effectively remove interfering odorless hydrocarbon compounds so that other odor-active compounds can be more easily identified (*13*). The second fraction (pentane:diethyl ether, 95:5) had weak fruity and apple-like notes, and was found to be rich of esters (Figure 2). Theaspirane A and B, 1-*p*-menthen-9-al were also included this fraction. The major aroma-active esters identified in this fraction were methyl hexanoate, ethyl hexanoate, and ethyl octanoate had typical fruit aroma (Table I). Esters constitute one of the largest groups of volatile compounds in many fruits. Compared with many other fruits, few esters were found and most of them were present only in trace levels in blackberry. The fractionation of esters makes it possible to identify the odor-active esters present at trace levels (*13*). Fraction 3 (pentane:diethyl ether, 90:10, Figure 3) contained aldehydes and ketones (Figure 3). This fraction had a fresh, green, fruity, and floral odor, and was enriched in ketones and aldehydes. Compounds such as hexanal, *cis*-3-hexenal, *trans*-2-hexenal, and octanal, seemed to contribute to the overall fresh and green aroma in this fraction, while 2-heptanone, β-damascenone, α-ionone, and β-ionone possessed the intense fruity and floral odor (Table I). The fourth fraction (pentane:diethyl ether, 50: 50) possessed strong fruity, spicy, and herbaceous notes, and was found to be concentrated in alcohols (Figure 4). Alcohols were one of the most predominant chemicals. Alcohols, such as 2-heptanol, *cis*-3-hexenol, *trans*-2-hexenol, linalool, benzyl alcohol, phenethyl alcohol, and 4-phenyl-2-butanol, had strong aroma reminiscent of fresh berry (Table I), which may contribute to the 'Black Diamond' aroma. The last fraction (methanol) had intensive floral, sweet odor and berry-like notes, and contained some very polar compounds sucha as hydroxyesters, terpinenediol, 3-hydroxy-β-damascone, 3-oxo-a-ionol, and 3-hydroxy-7,8-dihydro-β-ionol (Figure 5) compounds. The major aroma compounds identified in this fraction were lactones and furanones (Table I). Lactones seemed important for background odor because of their fresh, fruity aroma.However, lactones can be formed in the injector too. Mesifurane and furaneol contributed a positive aroma to 'Black Diamond'.

Silica gel fractionated the volatile compounds largely based on their functional groups, and greatly simplified the composition in each fraction. Only a few compounds appeared simultaneously in multiple fractions. The fractionation was very successful to help with peak identification and organoleptic recognition. The procedure allowed the identification of more than sixty aroma-active compounds in "Black Diamond" thornless blackberry (Table I).

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'Black Diamond' Aroma Constituents

Although fractionation provided information about the aroma quality of the compounds and positive mass spectrometry identification, this GC-O experiment could not provide a quantitative estimation of their odor intensity due to differences for the fractionations and concentration. A GC-O analysis for the mixture sample was more representative of the original 'Black Diamond' aroma.

Based on the GC/O analysis of the total mixture (Table II), the most significant (intensity >7) odor active volatiles in 'Black Diamond' were identified to be ethyl butanoate, ethyl hexanoate, 1-octen-3-one, 2-heptanol, *cis*-3-hexenol, nonanal, *trans*-2-hexenol, methional, linalool, ethyl 3-hydroxyhexanoate, α-ionone, β-ionone, furaneol, acetic acid, butanoic acid, and hexanoic acid.

Fresh 'Black Diamond' aroma has been described as fresh fruity, floral, strawberry, and raspberry notes (*14*), and the results matched this descriptive sensory evaluation. Ethyl butanoate, ethyl hexanoate, 1-octen-3-one, 2-heptanol, *cis*-3-hexenol, nonanal, *trans*-2-hexenol, methional, linalool, and ethyl 3-hydroxyhexanoate contributed to the fresh fruity and floral odor; α-ionone and β-ionone contributed to raspberry odor; furaneol contributed to strawberry odor. Typically, volatile acids are not considered as major aromas of fruit; acetic, butanoic, and hexanoic acid probably only accounted for background notes for 'Black Diamond'. However, among these high odor intensive aroma

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compounds, no one single compound was reminiscent of the 'Black Diamond' scent. Therefore, it is possible many other compounds, although with low odor intensities, could also contribute aroma to 'Black Diamond'. In addition, a few compounds, which had noticeable berry-like characteristics at the sniffing-port of the GC, and seemed important to 'Black Diamond' aroma, could not be identified. These compounds need to be further explored.

Overall, the identified aroma compositions were similar to other blackberry cultivars, especially 'Marion' blackberry (*8*). However, the odorants in different cultivars have various impacts. Different blackberry cultivars may share similar metabolic pathways such as fatty acid metabolism, amino acid metabolism, and carbohydrate metabolism. However, the specific pathways and enzyme activities may vary, resulting in a unique volatile composition and aroma profile of a specific variety.

In conclusion, despite the large number of volatiles positively identified, no single compound could be described as 'Black Diamond'-like. The 'Black Diamond' aroma is a complex mixture of aroma compounds acting in balance.

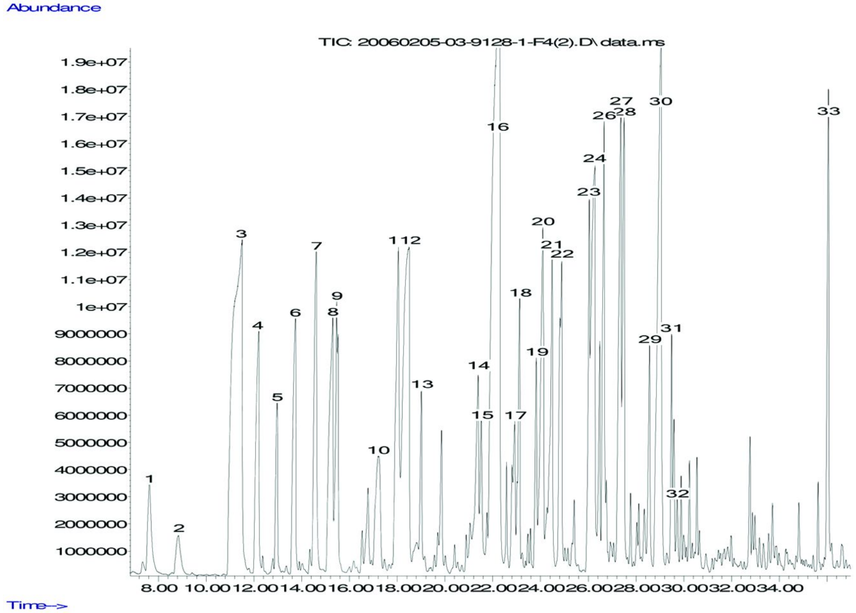

Figure 4. Chromatogram of fraction 4 (pentane:diethyl ether, 50: 50) obtained by normal-phase chromatography of neutral fraction of Black Diamond extract. Key: (1) 2-methylbutanol; (2) 3-methylbutanol; (3) 2-heptanol; (4) hexanol; (5) cis-3-hexenol; (6) trans-2-hexenol; (7) cis-linalool oxide; (8) heptanol; (9) trans-linalool oxide; (10) 2-nonanol; (11) linalool; (12) octanol; (13) mesifurane; (14) nonanol; (15) ethyl 3-hydroxyhexanoate; (16) α-terpineol; (17) 2-undecanol; (18) trans-epoxylinalool; (19) cis-epoxylinalool; (20) decanol; (21) myrtenol; (22) nerol; (23) p-cymen-8-ol; (24) geranol; (25) benzyl alcohol; (27) α-ionol; (28) phenethyl alcohol; (29) dihydro-β-ionone; (30) dihydro-β-ionol; (31) 4-phenyl-2-butanol; (32) perilla alcohol; (33) cinnamyl alcohol.

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Table II. Odor Active Compounds Identified in Black Diamond

Aroma compounds	*RI (Wax)*[a]	*RI (DB-5)*[b]	*Descriptors*	*Intensity*[c]	*Identication basis*
α-pinene	994	933	pine, resinous	6	MS, RI, aroma
ethyl butanoate	1015	788	rose, flower, fruity	8	MS, RI, aroma
camphene	1037	942	camphor	5	MS, RI, aroma
hexanal	1062	793	fruity, green	6	MS, RI, aroma
ethyl trans-2-butenoate	1151	845	sweet, flower, spicy	5	MS, RI, aroma
2-heptanone	1171	873	banana, floral, fruity	5	MS, RI, aroma
methyl hexanoate	1179	908	fruity, floral	6	MS, RI, aroma
limonene	1186	986	orange, citrus	6	MS, RI, aroma
ethyl hexanoate	1234	992	fruity, green	8	MS, RI, aroma
octanal	1294	1005	green, fatty, mushroom	6	MS, RI, aroma
1-octen-3-one	1303	968	mushroom, green	8	RI, aroma, Fr
2-heptanol	1339	898	moldy, mushroom	9	MS, RI, aroma
hexanol	1373	855	green, spicy, medical	6	MS, RI, aroma
trans-3-hexenol	1396	855	green, leaf	7	RI, aroma, Fr
cis-3-hexenol	1408	849	grassy, green	9	MS, RI, aroma
nonanal	1418	1098	citrus, fatty, hops, green	9	MS, RI, aroma
trans-2-hexenol	1437	852	green, leaf, walnut	8	MS, RI, aroma
cis-linalool oxide	1473	1032	sweet, woody, floral	7	MS, RI, aroma
acetic acid	1483	601	vinegar, sour	8	MS, RI, aroma
methional	1490	897	cooked potato	8	aroma, RIL
trans-linalool oxide	1508	1049	flower, floral	5	MS, RI, aroma
theaspirane A	1539	1242	woody, green, floral	4	MS, RI, aroma
ethyl 3-hydroxybutanoate	1573	965	green, fruity	6	MS, RI, aroma
theaspirane B	1585	1258	spicy, fruity, green	7	MS, RI, aroma
2-nonanol	1588	1188	fresh, green, fatty	5	MS, RI, aroma
linalool	1628	1069	citrus, green, sweet	8	MS, RI, aroma
octanol	1633	1038	moss, nut, green	5	MS, RI, aroma

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Table II. (Continued). Odor Active Compounds Identified in Black Diamond

Aroma compounds	*RI (Wax)*[a]	*RI (DB-5)*[b]	*Descriptors*	*Intensity*[c]	*Identication basis*
mesifurane	1663	-	sweet, maple, fruity	6	MS, RI, aroma
butanoic acid	1708	810	sour, rancid, cheesy	11	MS, RI, aroma
methyl 3-hydroxyhexanoate	1734	-	wine, fruity, oily	5	MS, RI, aroma
nonanol	1763	1186	fat, green, oily	6	MS, RI, aroma
2-methylbutanoic acid	1771	855	sour, salty, rancid	5	MS, RI, aroma
ethyl 3-hydroxyhexanoate	1780	1160	floral, rose	10	MS, RI, aroma
α-terpineol	1803	1150	anise, oily, mint	5	MS, RI, aroma
pentanoic acid	1862	-	goaty, woody, cheesy	6	MS, RI, aroma
ethyl salicylate	1939	1435	floral, fruity	5	MS, RI, aroma
nerol	1949	-	citrus, floral, green	7	MS, RI, aroma
dihydro-β-ionone	1977	-	tea, green, herbal	7	MS, RI, aroma
hexanoic acid	2010	1005	sweaty, sour, cheesy	8	MS, RI, aroma
benzyl alcohol	2040	998	fresh, tea, green	6	MS, RI, aroma
α-ionone	2050	1420	sweet, candy	8	RI, aroma, Fr
α-ionol	2071	-	fresh, tea, berry	6	MS, RI, aroma
benzeneethanol	2083	1113	floral, rose, honey	7	MS, RI, aroma
β-ionone	2110	1498	tea, violet	8	MS, RI, aroma
dihydro-β-ionol	2161	-	fresh, tea	6	MS, RI, aroma
3,7-dimethyloct-1-en-3,7-diol	2189	-	fresh, tea, berry	6	MS, RI, aroma
4-phenyl-2-butanol	2194	1214	green, tea	7	MS, RI, aroma
γ-nonalactone	2226	1365	coconut, sweet	6	MS, RI, aroma
furaneol	2250	1130	sweet, pineapple	8	RI, aroma, Fr
benzenepropanol	2261	1190	fatty, oil, berry	5	MS, RI, aroma
unknown	2277	-	berry, fruity, sweet	8	
ethyl cinnamate	2369	1471	sweet, berry, fruity	5	MS, RI, aroma
γ-decalactone	2383	1475	strawberry	6	MS, RI, aroma
unknown	2420	-	strawberry, tea, floral	8	

Continued on next page.

Table II. (Continued). Odor Active Compounds Identified in Black Diamond

Aroma compounds	*RI (Wax)*[a]	*RI (DB-5)*[b]	*Descriptors*	*Intensity*[c]	*Identication basis*
δ-decalactone	2445	1505	spicy, herbal	5	MS, RI, aroma
sotolon	2469	1115	maple, spicy, ceramel	7	MS, RI, aroma
vanillin	2963	1410	creamy, vanilla	6	MS, RI, aroma
Methyl vanillate	3016	1469	sweet, caramel, medical	5	MS, RI, aroma

[a] Retention index in ZB-WAX column; [b] Retention index in DB-5 column; [c] 16 point intensity scale was used where 0 = none, 3 = slight, 7 = moderate, and 15 = extreme intensities for each of descriptor

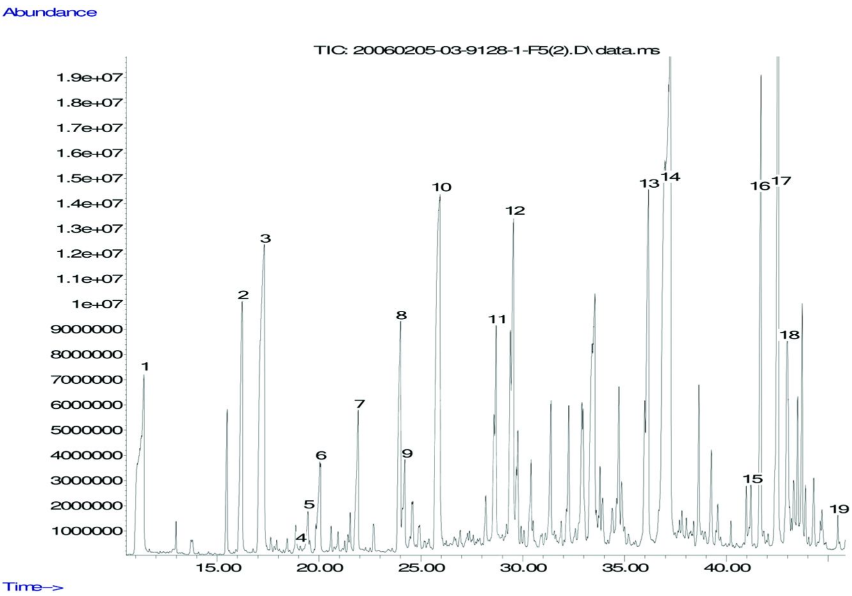

Figure 5. Chromatogram of fraction 5 (methanol) obtained by normal-phase chromatography of neutral fraction of Black Diamond extract. Key: (1) acetoin; (2) methyl 3-hydroxybutanoate; (3) ethyl 3-hydroxybutanoate; (4) mesifurane; (5) γ-valerolactone; (6) butyrolactone; (7) γ-hexalactone; (8) methyl 4-hydroxybutanoate; (9) δ-hexalactone; (10) methyl 5-hydroxyhexanoate; (11) terpendiol; (12) 3,7-dimethyloct-1-en-3,7-diol; (13) 8-acetoxylinalool; (14) hydroxylinalool; (15) 3-hydroxy-β-damascone; (16) vanillin; (17) methyl vanillate; (18) 3-oxo-a-ionol; (19) 3-hydroxy-7,8-dihydro-β-ionol.

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References

1. Finn, C. E.; et al. 'Black Diamond' trailing thornless blackberry. *HortScience* **2005**, *40*, 2175–2178.
2. Scanlan, R. A.; Bills, D. D.; Libbey, L. M. Blackberry flavor components of commercial essence. *J. Agric. Food Chem.* **1970**, *18* (4), 744.
3. Gulan, M. P.; et al. Compounds identified in commercial blackberry essence. *J. Agric. Food Chem.* **1973**, *21* (4), 741.
4. Georgilopoulos, D. N.; Gallois, A. N. Aroma compounds of fresh blackberries (*Rubus laciniata* L.). *Z. Lebensm. Unters. Forsch.* **1987**, *184* (5), 374–380.
5. Georgilopoulos, D. N.; Gallois, A. N. Volatile flavor compounds in heated blackberry juices. *Z. Lebensm. Unters. Forsch.* **1987**, *185* (4), 299–306.
6. Georgilopoulos, D. N.; Gallois, A. N. Flavor compounds of a commercial concentrated blackberry juice. *Food Chem.* **1988**, *28* (2), 141–148.
7. Klesk, K.; Qian, M. C. Preliminary aroma comparison of Marion (Rubus spp. hyb) and Evergreen (*R. laciniatus* L.) blackberries by dynamic headspace/ OSME technique. *J. Food Sci.* **2003**, *68* (2), 697–700.
8. Klesk, K.; Qian, M. C. Aroma extract dilution analysis of cv. Marion (Rubus spp. hyb) and cv. Evergreen (*R. laciniatus* L.) blackberries. *J. Agric. Food Chem.* **2003**, *51* (11), 3436–3441.
9. Qian, M. C.; Wang, Y. Y. Seasonal variation of volatile composition and odor activity value of Marion (Rubus spp. hyb) and Thornless Evergreen (*R. laciniatus* L.) blackberries. *J. Food Sci.* **2005**, *70* (1), C13–C20.
10. Qian, M. C.; Burbank, H. M.; Wang, Y. Y. Preseparation techniqies in aroma analysis. In *Sensory-direct Flavor Analysis*; Marsili, R., Ed.; CRC Press: Boca Raton, FL, 2006; p 288.
11. Wang, Y. Y.; Finn, C.; Qian, M. C. Impact of growing environment on Chickasaw blackberry (Rubus L.) aroma evaluated by gas chromatography olfactometry dilution analysis. *J. Agric. Food Chem.* **2005**, *53* (9), 3563–3571.
12. Qian, M.; Reineccius, G. Identification of aroma compounds in Parmigiano-Reggiano cheese by gas chromatography/olfactometry. *J. Dairy Sci.* **2002**, *85* (6), 1362–1369.
13. Qian, M. C.; Burbank, H. M.; Wang, Y., Preseparation techniques in aroma analysis. In *Sensory-Directed Flavor Analysis*; Marsili, R., Ed.; CRC Press LLC: Boca Raton, FL, 2007; pp 111−154.
14. Kurnianta, A. J. Descriptive sensory analysis of thornless blackberry selections to determine sensory similarity to 'Marion' blackberry flavor. *Food Science and Technology*; Oregon State University: Corvallis, OR, 2005.

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Chapter 5

Aroma-Impact Components of "Carlos" Muscadine Grape Juice

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Characteristic aroma components of Carlos muscadine grape juice were identified and quantified by gas chromatography-olfactometry (GCO) and GC-MS techniques. Combined results of GCO, quantitative analysis and calculated odor-activity values (OAVs) revealed seven compounds as potent odorants, namely ethyl 2-methylbutanoate*y*, ethyl 3-methylbutanoate, 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone, 2-phenylethanol, ethyl hexanoate, ethyl (*E*)-2-butenoate and *o*-aminoacetophenone. (*E*)-β-Damascenone and 3-hydroxy-4,5-dimethyl-2(*5H*)-furanone were also indicated as important odorants by AEDA, but these compounds were below GC-MS detection limits and, thus, OAVs were not calculated. A sensory aroma profile comparison between authentic muscadine grape juice and a model juice composed of 20 odorants revealed the model contained comparable intensities for musky, caramel and fruity notes, but lower intensities for the overripe, floral and sour notes. The sensory profile of the model was adjusted to better approximate the authentic juice by increasing the levels of 3-methylbutanoic acid and 2-phenylethanol and by the addition of (*E,Z*)-2,6-nonadien-1-ol.

The muscadine grape (*Vitis rotundifolia* Michx) is native to the Southeastern United. 'Carlos' is a popular bronze variety and is used in production of several

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commercial products including juice, wine, and jelly. Because of their typical/characteristic muscadine flavors, the juice and jelly products are highly favored, while muscadine wines are considered to be inferior to those made from *Vitis vinifera* varieties.

Several reports on the volatile flavor constitutions of muscadine grapes have been published (*1–5*). Researchers have identified the predominant aroma compounds in muscadine grape juice by gas chromatography-olfactometry (GCO) and aroma extract dilution analysis (AEDA) (*4*). The semivolatile compounds 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone (Furaneol™) and o-aminoacetophenone were shown to be responsible for characteristic candy and foxy-like aroma notes, respectively, in muscadine (*4*). However, little information is available regarding the contribution of highly volatile headspace compounds to the aroma of muscadine grape juice.

Solvent extraction has been used for the isolation of the volatile constituents of muscadine grape juice (*4–7*). However, this technique has a major limitation since highly volatile headspace components may be fully or partially lost during concentration and workup of the solvent extracts. In order to determine the impact of highly volatile aroma components on the aroma of muscadine grape juice, application of complementary volatile sampling methods is required, such as use of both solvent extraction and static headspace analysis.

The objective of the present study was to apply instrumental and sensory techniques to determine the overall contribution of highly volatile and semivolatile constituents to the characteristic aroma of 'Carlos' muscadine grape juice.

Experimental

Materials

Muscadine (cultivar 'Carlos') grapes were obtained from the Mississippi Agriculture and Forestry Experiment Station (MAFES) Truck Crops Branch (Crystal Springs, MS) and frozen at -18 °C until processed into juice as earlier described (*4*). Juice was bottled in 250-mL glass containers equipped with PTFE-lined caps and stored at -20 °C. Before analysis, juice was thawed by placing the glass container in a water bath (25 °C) for 2 h.

All reagents and authentic flavor compounds were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO), except for *p*-vinylguaiacol (Lancaster Synthesis, Inc.; Windham, NH) and (*E*)-β-damascenone (Firmenich Inc.; Plainsboro, NJ). Dichloromethane was redistilled prior to use. Deodorized distilled water was prepared by boiling distilled water until the contents were decreased by one-third of the original volume.

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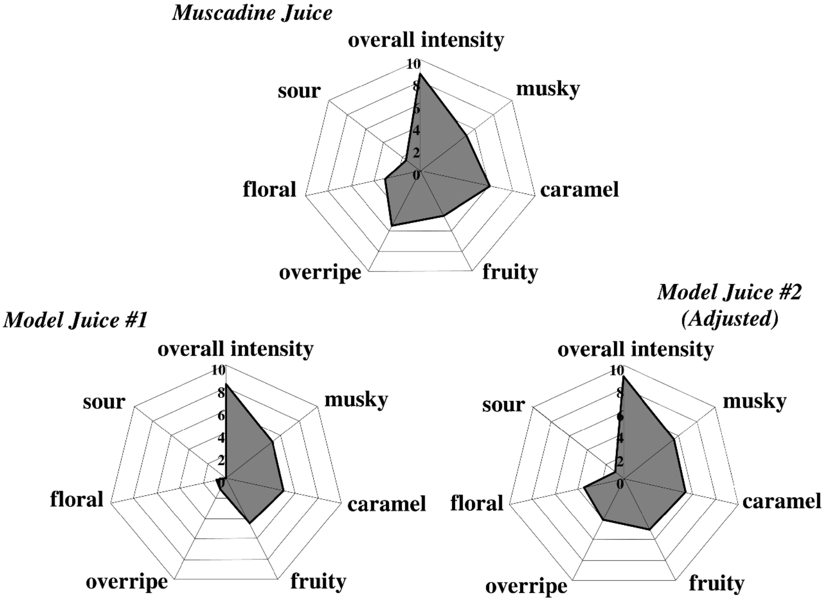

Figure 1. Sensory aroma profiles of muscadine and model juices.

Composition Analysis

pH, Titratable Acidity, and Soluble Solids

pH was measured using a model 250A pH meter equipped with a model 9107EN pH electrode (Orion; Boston, MA). Titratable acidity (TA) was determined using a published procedure and expressed as percent tartaric acid (*8*). A hand held refractometer (Fisher Scientific; Pittsburgh, PA) was used to measure percent soluble solids.

Sugars

High performance liquid chromatography (HPLC) was used to analyze fructose, glucose, and sucrose. The system consisted of a model 110B Pump (Beckman; Fullerton, CA) equipped with a Rheodyne manual injector and a Varian RI-4 refractive index detector (Varian Inc., Walnut Creek, CA) and a HP3390A integrator (Agilent Technologies, Inc., Palo Alto, CA). The filtered (0.45 μm cellulose acetate membrane) sample was injected (5 μL sample loop) and separations performed on an HPX-87P Aminex resin-based column (300 mm x 7.8 mm i.d., cat. no. 125-0098, Bio-Rad, South Richmond, CA) equipped with an HPX-87P guard cartridge (30 mm x 7.8 mm i.d. cat. No. 125-0119, Bio-Rad). HPLC-grade water was used as mobile phase at a flow rate of 0.6 mL/min and a column temperature of 85°C. Concentrations were calculated from peak areas as compared to standard curves (sucrose at 0.050, 0.075, 0.100, 0.150 and 0.200% (w/v); fructose and glucose at 0.25, 0.50, 0.75, 1.00 and 1.25% (w/v) prepared in HPLC-grade water).

Organic Acids

Individual acids were determined by HPLC using a Vista 5500 system (Varian Associates, Walnut Creek, CA) equipped with a UV detector set at 210 nm. Filtered juice was injected into the HPLC system (10 µL sample loop) for analysis of malic acid and tartaric acids. Separations were performed on an HPAH 1414 Fast Acid Analysis column (7.8 mm i.d. x 100 mm, cat. no. 125-0100, Bio-Rad) with aqueous 0.005M H_2SO_4 as the mobile phase at a flow rate of 0.2 mL/min. Column temperature was held at 30°C. Peak integration was done by using a HP3395 integrator (Agilent Technologies, Inc.). Calibration plots were generated for malic acid and tartaric acid by analyzing solutions (prepared in mobile phase) of 0.0125, 0.025, 0.050 and 0.100 (w/v).

Pectin

Pectin was assayed in duplicate according to the procedure of Robertson (*9*).

Determination of Volatile Constituents

Liquid-Liquid Continuous Extraction

A liquid-liquid continuous extractor (LLCE) (cat. no. 291650-0000, Kontes, Vineland, NJ.) was used to extract the volatile compounds from the grape juice. The juice was acidified to pH 2 with aqueous 0.5N HCl prior to extraction. Muscadine juice (12 mL) plus 3 mL of deodorized distilled water and 5 µg of 3-heptanol (internal standard) were placed in the extractor. Dichloromethane (25 mL) was used as the extracting solvent. Extractions were carried out for 16 h at room temperature (~ 23-25° C). The extract was frozen at -20 °C overnight to remove excess water as ice crystals. The volume of extract was concentrated to 10 mL under a gentle stream of nitrogen, dried over 1 g of anhydrous sodium sulfate. Extracts were concentrated to 20 µL prior to analysis. Extractions were performed in duplicate and extracts were stored at -20 °C in amber glass vials equipped with Teflon-lined screw caps.

Aroma Extract Dilution Analysis (AEDA)

GC-olfactometry (GCO) system consisted of a Varian 3300 GC (Varian, Inc.) equipped with a flame ionization detector (FID) and a sniffing port. Serial dilutions

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(1:3) of extracts were prepared using dichloromethane as a diluent. From each dilution 1 µL was injected (splitless mode) into a capillary column (DB-WAX or DB-5MS, 30 m length x 0.32 mm i.d. x 0.25 µm df; J & W Scientific, Folsom, CA). GC conditions were the same as GC-MS except that oven temperature was programmed from 40 to 200 °C at a ramp rate of 8 °C/min with initial and final hold times of 5 and 30 min, respectively. FID and the sniffing port transfer line were held at 250 °C. Other details of GCO have been described by Baek and Cadwallader (*4*). The highest at which an odorant was detected by GCO was defined as its flavor dilution (FD)-factor (*10*).

Gas Chromatography-Olfactometry of Decreasing Headspace Samples (GCO-H)

Muscadine grape juice (10 mL) was placed into a 250-mL round bottom flask and the flask sealed with a Teflon-lined septum. The flask was incubated in a 40 °C water bath for 30 min to allow the volatile compounds to partition into the headspace of the flask and to reach gas phase equilibrium concentrations. A headspace volume (0.1, 0.5, 1, 5 or 10 mL) was withdrawn from the flask using a preheated (60 °C) gastight syringe and then immediately injected into an HP 5980 GC (Agilent Technologies, Inc.) equipped with a packed column inlet modified for capillary GC injection (Uniliner Sleeve Adapter, Resteck Corp., Bellefonte, PA) at a flow rate of 5 mL/min. Separations were performed on DB-MS and DB-WAX columns (30 m length x 0.53 mm i.d. x 1.5 µm (or 1 µm for DB-WAX) film thickness; J&W Scientific, Folson, CA). Prior to and during injection, a 15-cm section of the column was cooled in liquid nitrogen in order to cryofocus the volatiles at the head of the GC column. After injection, the GC was rapidly heated and the run immediately started when oven temperature reached 40 °C. Oven temperature was programmed from 40 to 200 °C at a ramp rate of 10 °C/min, with initial hold time of 5 min and final hold time of 20 min. FID and sniffing port transfer line were held at 250 °C. GCO-H data were analyzed according using the procedure described by Zhou et al. (*11*).

Gas Chromatography-Mass Spectrometry (GC-MS).

GC-MS analysis was performed using an HP 5890 Series II GC/HP 5972 mass selective detector (MSD) system (Agilent Technologies, Inc.). Extract (2 µL) was injected (200 °C, splitless mode, 30 s valve-delay) into a fused silica capillary column (DB-WAX or DB-5, 60 m length x 0.25 mm i.d. x 0.25 µm film thickness, J&W Scientific, Folsom, CA). Helium was the carrier gas at a constant flow rate of 1.0 mL/min (linear velocity of 25 cm/s). Oven temperature was programmed from 40°C to 200°C at a ramp rate of 3 °C /min with initial and final hold times of 5 and 60 min, respectively. MSD conditions were as follow: capillary direct interface temperature, 280 °C; ionization energy, 70 eV; mass range, 33-350 a.m.u.; electron multiplier (EM) voltage, 200V above autotune; scan rate, 2.2 scans/s.

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Compound Identification.

Positive compound identifications were based on comparison of retention indices (RI) (*12*), mass spectra, and odor properties of unknowns with those of authentic standard compounds analyzed under identical experimental conditions. Tentative identifications were based on matching RI values and odor properties of unknowns with those of authentic standards or on matching mass spectra against those in a database (*Wiley 138*, John Wiley and Sons, Inc., 1990).

Dynamic Headspace Sampling (DHS)-GC-MS.

Muscadine grape juice (10 mL) plus internal standard (5 µg of 3-heptanol) was placed in a 25-mL headspace sampling tube (15.2 cm × 1.6 cm i.d.) of a GCO-DHS system consisting of a Tekmar 3000 Purge and Trap (P&T) Concentrator/Cryofocusing Module (Tekmar Co., Cincinnati, OH) coupled with an HP 5890 Series II GC/HP 5972 mass selective detector (MSD) system (Agilent Technologies, Inc.). The volatiles were purged at 60°C with helium (40 mL/min) for 10 min onto to a Tenax TA trap (part no. 12-0083-303, Tekmar Co.) maintained at 0 °C. After sampling, the trap was dry purged for 5 min and then the volatiles were desorbed (180°C for 1 min) and subsequently cryofocused (-120 °C) onto a 15 cm section of 0.53 mm i.d. deactivated fused silica capillary column. Transfer lines and valves were maintained at a temperature of 175°C. Trap pressure control was set at 4 psi during purging. Helium flow during thermal desorption of Tenax trap (20 mL/min) and cryofocusing trap (1.4 mL/min) was controlled by the split/splitless electronic pressure control pneumatics of the GC. Cryofocused volatiles were thermally desorbed (180°C for 1 min) directly into the analytical GC column. Between each analysis, the system was cleaned by purging with clean glassware installed and Tenax TA trap subsequently baked (225°C for 10 min). The other conditions of GC-MS were the same as earlier described.

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Quantitative Analysis

Preparation of Juice Matrix

Juice matrix was prepared by dissolving sucrose (2.26 g), glucose (72.26 g) and fructose (87.28 g), malic acid (3.91 g), tartaric acid (3.59 g) and pectin (1.95 g) in 1 L of deodorized tap water.

Calibration Curves

A standard stock solution containing 10 aroma compounds was prepared in the juice matrix. Stock solution was diluted to 1:2, 1:4 and 1:8 to make the working standard solutions. Forty mL of stock standard flavor solution was added to 48 mL of juice matrix in a 250-mL glass bottle equipped with screw cap. After

5 min shaking, 40 mL of mixture was acidified to pH 2 by 0.5 N HCl then the mixture was decanted into a 50-mL volumetric flask and made to the volume with deodorized distilled water. For solvent extraction, an aliquot of the mixture (15 mL, equivalent to 12 mL of model juice plus 3 mL of deodorized distilled water) was spiked with internal standard (5 µg of 3-heptanol) and extracted by LLCE and analyzed by GC-MS as previous described. For DHS-GC-MS standard solutions were made up in juice matrix and 10 mL aliquots were spiked with internal standard (2.04 µg of 2-methyl-3-heptanone) before analysis. Standard curves of peak area ratio (compound/internal standard) versus mass ratio (compound/internal standard) were prepared for each aroma compound and were used to calculate the concentrations of the aroma compounds in the muscadine juice.

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Sensory Studies

Juice Models

Juice model #1 was constructed by adding the following 20 compounds, diluted in 95% ethanol, to 1 L of the juice matrix: methyl acetate (3060 µg), ethyl acetate (32600 µg), ethyl propionate (170 µg), ethyl 2-methylpropanoate (1 µg), 2-methylpropyl acetate (49 µg), ethyl butanoate (40µg), ethyl 2-methylbutanoate (80 µg), ethyl 3-methylbutanoate (128 µg), 3-methyl-1-butanol acetate (950 µg), ethyl (E)-2 butenoate (3960 µg), 3-methyl-1-butanol (1260 µg), ethyl hexanone (196 µg), acetate acid (751170 µg), ethyl 3-hydroxybutanone (930 µg), 3-methylbutanoic acid (200 µg), phenethyl acetate (7 µg), 2-phenylethanol (900 µg), 4-hydroxy-2,5-dimethyl-3(2H)furanone (8410 µg), p-vinylguaiacol (20 µg), and *o*-aminoacetophenone (20 µg). Juice model #2 was the same as model #1 except for higher concentrations of 3-methylbutanoic acid (400 µg) and 2-phenylethanol (1800 µg) and for the addition of (*E,Z*)-2,6-nonadien-1-ol (5 µg).

Descriptive Analysis

Sensory descriptive analysis was conducted by a 12-person trained panel consisting of staff and students of the MSU Department of Food Science and Technology. Panelistis had previously participated in formal sensory training and received an additional 4 h of training with muscadine grape juice where they defined aroma (odor) terms, including musky, caramel, fruity, overripe, floral and sour. Aroma attributes were evaluated orthonasally using 125-mL PTFE squeeze bottles containing 10 mL of juice. Panelists were instructed to score the overall aroma and individual aroma attributes using a 15-point universal scale, where 0 = none and 15 = very strong (*13*). Other procedural details and statistical analyses have been previously described (*12*).

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Results and Discussion

Non-Volatile Composition

An important first step for the accurate formulation of a model muscadine juice system was the development of a well-defined nonvolatile juice matrix. Grape juice is composed mainly of sugars, organic acids and pectin (*14–16*). The nonvolatile composition of the 'Carlos' muscadine grape juice used in the present study is given in Table I. Fructose, glucose and sucrose were the major sugars, while the principal organic acids were tartaric and malic acids. Pectin content, expressed as polygalaturonic acid, was 0.195 g per 100 mL of juice. These findings are in agreement with previous reports on muscadine juice composition (*17*).

Aroma-Active Components

In a previous study, Baek et al. (*4*) determined the major aroma-impact components of 'Carlos' muscadine grape juice. They used liquid-liquid continuous extraction (LLCE) to isolate the volatile constituents from three different juice samples and the aroma-active components were determined by gas chromatography-olfactometry (GCO) and aroma extract dilution analysis (AEDA). Their results indicated that 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone, 2,3-butanedione, ethyl butanoate, ethyl 2-methylbutanoate, 2-phenylethanol and *o*-aminoacetophenone were the predominant aroma components of 'Carlos' muscadine juice. In the present study, a small-scale LLCE technique was used to isolate the volatile components of a 'Carlos' muscadine juice. GCO and AEDA results showed that this juice had essentially the same predominant aroma components (Table II). Nine additional compounds were identified in the present study (nos. **6**, **11**, **12**, **17**, **21**, **22**, **24**, **28**, **30** and **36**) which had not been previously reported by Baek et al. (*4*). The compound β-damascenone (no. **28**) was previously identified as a minor volatile component of muscadine grape (*6*).

There was some uncertainty as to whether AEDA allowed for the analysis of all important aroma components of the juice. While AEDA is an excellent technique for the identification of odorants of intermediate and low volatility, highly volatile headspace aroma compounds, such a low molecular weight aldehydes and esters, might be lost during the solvent extraction and concentration steps. Therefore, to complement AEDA, GCO of decreasing static headspace samples (GCO-H) was used to analyze the highly volatile aroma-active constituents of the juice. GCO-H revealed a total of 23 aroma-active components (Table III). Among these, compounds nos. **1-5**, **7**, **9** and **13** were not detected by AEDA in the present study. Meanwhile, 16 compounds (nos. **6**, **10-12**, **15-17**, **19**, **21**, **22**, **28**, **29**, **32**, **34**, **36** and **37**) were detected by both AEDA and GCO-H.

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Table I. Nonvolatile Composition of 'Carlos' Muscadine Grape Juice

pH	3.21	
soluble solids (°Brix)	16.0	
titratable acidity (as tartaric acid, g/100 mL)	0.76[a]	
		Cncn (g/100 mL)[a]
glucose		7.23
fructose		8.73
sucrose		0.226
malic acid		0.391
tartaric acid		0.359
pectin		0.195

[a] Average (n = 2).

Quantitative Results and Odor Activity Values (OAVs)

OAVs can be used to complement or verify the results of GCO techniques. Quantitative results and OAVs for twenty selected volatile components of 'Carlos' muscadine juice are given in Table IV. Based on the OAVs, the major contributors to the aroma of 'Carlos' muscadine juice were ethyl 3-methylbutanoate (no. **11**), 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone (no. **34**), ethyl 2-methylbutanoate (no. **10**), ethyl (*E*)-2-butenoate (no. **9**), ethyl hexanoate (no. **15**), *o*-aminoacetophenone (no. **37**), 2-phenylethanol (no. **32**) and ethyl butanoate (no. **8**). 4-Hydroxy-2,5-dimethyl-3(*2H*)-furanone and *o*-aminoacetophenone are important characterizing aroma components which impart caramel and musky notes, respectively, (*18*, *19*) which are reminiscent of the characteristic aroma of muscadine juice. The esters contributed fruity and candy-like notes, while *o*-aminoacetophenone and 2-phenylethanol imparted musky and floral notes, respectively.

Aroma Profile Comparison of 'Carlos' Muscadine Grape and Model Juices

One way of determining the actual importance of individual aroma compounds in the overall aroma profile of 'authentic' juice is to employ sensory evaluation to compare the aroma profile of the authentic juice with that of a model juice system. In the present study, the model juice system was developed based on the analytical data in Table IV and the nonvolatile matrix composition given in Table I. The sensory aroma profile 'Carlos' muscadine juice sample is shown in Figure 1. Some links can be made between the sensory terms and the potent odorants in Tables II−IV. For example, the caramel attribute may be due to 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone (no. **34**), the musky note due to *o*-aminoacetophenone (no. **37**), floral note to 2-phenylethanol (no. **32**),

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and sour note due to acetic acid (no. **18**) and 3-methylbutanoic acid (no. **25**). The esters collectively accounted for the fruity note, whereas the overripe fruit note could have been due to the presence of (*E*)-β-damascenone (no. **28**) and (*E,Z*)-2,6-nonadienal (ol) (nos. **23** and **27**, respectively).

Table II. Odorants Detected by Aroma Extract Dilution Analysis of 'Carlos' Muscadine Grape Juice

No.[a]	*compound*	*odor description*[b]	*retention index*[c]		*Log_3FD-factor*[d]	
			WAX	*DB5*	*WAX*	*DB5*
6	ethyl 2-methylpropanoate[e]	berry, fruity	965	762	<1	<1[f]
8	ethyl butanoate[e]	bubble gum, fruity	1027	802	<1	<1
10	ethyl 2-methylbutanoate[e]	berry, fruity	1056	850	<1	1
11	ethyl 3-methylbutanoate[e]	berry, fruity	1069	856	<1	<1
12	(*Z*)-3-hexenal[e]	green, cut-leaf	1127	798	1	<1
14	3-methyl-1-butanol[e]	malty, dark chocolate	1220	- -[g]	1	n.d.[h]
15	ethyl hexanoate[e]	apple, friuty	1240	998	<1	<1
16	1-octen-3-one[e]	mushroom	1315	- -	<1	n.d.
17	(*Z*)-3-hexen-1-ol[e]	green, cut-leaf	1391	855	1	<1
18	acetic acid[e]	vinegar, sour	1444	n.a.	3	n.d.
19	3-(methylthio)propanal[i]	potato	1453	912	n.d.	<1
20	ethyl 3-hydroxybutanoate[e]	marshmallow, fruity	1524	935	<1	<1
21	(*E*)-2-nonenal[i]	hay-like, fatty	1533	1145	1	1
22	linalool[e]	floral	1561	1261	<1	2
23	(*E,Z*)-2,6-nonadienal[i]	fresh, cucumber, melon	1582	1160	<1	<1
24	mercenol[j]	gasoline, musky	1616	1017	1	<1
25	3-methylbutanoic acid[e]	sweaty	1672	854	2	<1
26	*unknown*	savory, saffron	1730	- -	1	n.d.
27	(*E,Z*)-2,6-nonadien-1-ol[i]	cucumber, melon	1771	1157	1	<1
28	β-damascenone[e]	applesauce	1825	1389	1	<1
29	*unknown*	stewish, sour cherry	1837	- -	2	n.d.
30	guaiacol[e]	smoky	1860	1091	<1	<1
31	*unknown*	grape, floral	1892	1361	1	<1
32	2-phenylethanol[e]	rosy, wine-like	1906	1120	5	3

Continued on next page.

Table II. (Continued). Odorants Detected by Aroma Extract Dilution Analysis of 'Carlos' Muscadine Grape Juice

No.[a]	*compound*	*odor description*[b]	*retention index*[c]		*Log$_3$FD-factor*[d]	
			WAX	*DB5*	*WAX*	*DB5*
33	*unknown*	planty, tomato leaf	- -	1126	n.d.	<1
34	4-hydroxy-2,5-dimethyl-3(2H)-furanone (Furaneol™)[e]	burnt sugar, strawberry	2036	1062	7	5
35	*p*-vinylguaiacol	smoky, cloves	2196	1364	2	1
36	3-hydroxy-4,5-dimethyl-2(*5H*)-furanone (sotolon)[i]	curry, spicy	2197	1112	5	<1
37	*o*-aminoacetophenone[e]	grape, musky	2208	1315	6	4

[a] Numbers correspond to those in Tables III and IV. [b] Odor quality as perceived during GCO. [c] Retention indices were calculated from GCO data. [d] Log$_3$Flavor dilution (FD) factor. [e] Compound positively identified based on comparison of its mass spectrum, odor property and retention indices with reference compound. [f] Compound detected in concentrated extract only. [g] - - = not available. [h] n.d. = not detected. [i] Compound tentatively identified based on comparison of its odor property and retention indices with reference compound. [j] Compound tentatively identified based on comparison of its mass spectrum to MS database.

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Table III. Odorants Detected by Gas Chromatography-Olfactometry of Decreasing Headspace Volumes of 'Carlos' Muscadine Grape Juice

No.[a]	*compound*	*odor description*[b]	*retention index*[c]		*FD-factor*[d]	
			WAX	*DB5*	*WAX*	*DB5*
1	acetaldehyde[e]	yogurt, pungent	650	<500	20	20
2	methylpropanal[e]	dark chocolate	822	529	10	10
3	ethyl acetate[e]	solvent, nail polish	885	603	2	2
4	3-methylbutanal[e]	dark chocolate	907	644	2	1
5	ethanol[e]	ethanolic	947	- -[f]	20	n.d.[g]
6	ethyl 2-methylpropanoate[e]	berry, fruity	965	762	20	10
7	2,3-butanedione[h]	buttery, cream cheese	986	614	1	n.d.
9	ethyl(*E*)-2-butenoate	green apple, fruity	1049	848	20	100
10	ethyl 2-methylbutanoate[e]	berry, fruity	1056	850	10	
11	ethyl 3-methylbutanoate[e]	berry, fruity	1069	856	2	2
12	(*Z*)-3-hexenal[e]	green, cut-leaf	1127	800	2	n.d.

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Table III. (Continued). Odorants Detected by Gas Chromatography-Olfactometry of Decreasing Headspace Volumes of 'Carlos' Muscadine Grape Juice

No.[a]	*compound*	*odor description*[b]	*retention index*[c]		*FD-factor*[d]	
			WAX	*DB5*	*WAX*	*DB5*
13	1,8-cineol	minty, eucalyptus	1211	- -	1	n.d.
15	ethyl hexanoate[e]	apple, fruity	1240	998	1	10
16	1-octen-3-one[h]	mushroom	1315	979	1	2
17	(*Z*)-3-hexen-1-ol[e]	green, cut-leaf	1391	855	n.d.	2
19	3-(methylthio)propanal[h]	Potato	1453	912	n.d.	2
21	(*E*)-2-nonenal[h]	hay-like, fatty	1533	1145	1	n.d
22	linalool[e]	Floral	1561	1261	1	n.d.
28	β-damascenone[e]	applesauce	1825	1389	20	1
29	*unknown*	stewish, sour cherry	1837	- -	2	n.d.
32	2-phenylethanol[e]	rosy, wine-like	1906	1120	2	2
34	4-hydroxy-2,5-dimethyl-3(2H)-furanone (Furaneol™)[e]	burnt sugar, strawberry	2036	1062	100	2
36	3-hydroxy-4,5-dimethyl-2(*5H*)-furanone (sotolon)[h]	curry, spicy	2197	1112	1	2
37	*o*-aminoacetophenone[e]	grape, musky	2208	1315	20	10

[a] Numbers correspond to those in Tables II and IV. [b] Odor quality as perceived during GCO. [c] Retention indices were calculated from GCO data. [d] Flavor dilution (FD) factor calculated by dividing the highest headspace volume tested (10 mL) by the lowest headspace volume required for detection of a compound (i.e., 10, 5, 1, 0.5 or 0.1 mL). [e] Compound positively identified based on comparison of its mass spectrum, odor property and retention indices with reference compound. [f] - - = not available. [g] n.d. = not detected. [h] Compound tentatively identified based on comparison of its odor property and retention indices with reference compound.

Table IV. Concentrations, Odor Thresholds and Odor-Activity Values for Selected Aroma Components of 'Carlos' Muscadine Grape Juice

No.[a]	*compound*	*Cncn (μg/L)*[b]	*Threshold (μg/L) in water*[c]	*OAV*[d]
- -	methyl acetate	3060	- -[e]	- -
3	ethyl acetate	32600	12200	3
- -	ethyl propanoate	170	10	17

Continued on next page.

Table IV. (Continued). Concentrations, Odor Thresholds and Odor-Activity Values for Selected Aroma Components of 'Carlos' Muscadine Grape Juice

No.[a]	*compound*	*Cncn (μg/L)*[b]	*Threshold (μg/L) in water*[c]	*OAV*[d]
6	ethyl 2-methylpropanoate	1.33	0.1	13
- -	2-methylpropyl acetate	49.1	n.a.	- -
8	ethyl butanoate	41.4	1	41
9	ethyl (*E*)-2-butenoate	3960	13.6	291
10	ethyl 2-methylbutanoate	79.5	0.15	530
- -	3-methyl-1-butanyl acetate	951	19	50
11	ethyl 3-methylbutanoate	128	0.2	640
14	3-methyl-1-butanol	1260	250	5
15	ethyl hexanoate	196	1	196
18	acetic acid	751000	50000	1.5
20	ethyl 3-hydroxybutanoate	930	- -	- -
25	3-methylbutanoic acid	202	250	<1
- -	phenethyl acetate	7.01	20	<1
32	2-phenylethanol	902	17[f]	53
34	4-hydroxy-2,5-dimethyl-3(*2H*)-furanone	8410	21[g]	400
35	p-vinylguaiacol	20.5	20	1
37	*o*-aminoacetophenone	20.6	0.2	103

[a] Numbers correspond to those in Tables II and III. [b] Average concentration (μg/L)(n = 2). [c] Odor detection thresholds in water from reference (*20*). [d] Odor-activity value calculated by dividing concentration by odor detection threshold. [e] - - = not available. [f] Odor detection threshold in water from reference (*21*). [g] Odor detection threshold in water at pH 3 from reference (*22*).

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Further sensory experiments were performed to establish whether or not compounds having high OAVs are actually essential to the characteristic muscadine juice aroma. First, the 20 aroma compounds listed in Table IV were dissolved in model juice matrix and the final pH adjusted to the same level as the authentic juice (Table I). The aroma profile comparison of this model juice #1 system with the 'real' juice is shown in Figure 1. Intensities for musky, caramel and fruity aroma attributes were similar for the two aroma profiles. Meanwhile the overripe, floral and sour notes were scored at lower intensities in the model juice.

In the second experiment, concentrations of some of the aroma compounds in model juice #1 were adjusted to produce model juice #2, which contained higher levels of 3-methylbutanoic acid and 2-phenylethanol. Furthermore, (*E,Z*)-2,6-nonadien-1-ol was included in model juice #2 in an attempt to increase the overripe note. These adjustments caused the aroma profile model #2 to more closely resemble that of the authentic muscadine juice (Figure 1).

Conclusions

Results of this study revealed the odorants responsible for the overall aroma of 'Carlos' muscadine grape juice. Based on the results of GCO, quantitative analysis and calculation OAVs, and sensory experiments confirmed the importance of 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone, *o*-aminoacetophone, 2-phenylethanol, ethyl 2-methylbutanoate and ethyl butanoate as key odorants in the overall aroma of muscadine juice. In addition to the above mentioned compounds, several additional esters (e.g., ethyl 3-methylbutanoate , ethyl (*E*)-2-butenoate and ethyl hexanoate) were shown to be important contributors to the fruity character of muscadine juice. The above mentioned compounds may serve as useful indicators for the objective measurement of flavor changes caused by raw materials, processing conditions and storage of muscadine grape products.

References

1. Kepner, R. E.; Webb, A. D. Volatile aroma constituents of *Vitis rotundifolia* grapes. *Am. J. Enol. Vitic.* **1956**, *7*, 8–18.
2. Welch, R. C.; Johnston, J. C.; Hunter, G. L. K. Volatile constituents of the muscadine grape (*Vitis rotundifolia*). *J. Agric. Food. Chem.* **1982**, *30*, 681–684.
3. Horvat, R. J.; Senter, S. D. Identification of the volatile constituents from scuppernong berries (*Vitis rotundifolia*). *J. Food Sci.* **1984**, *49*, 64–66, 81.
4. Baek, H. H.; Cadwallader, K. R.; Marroquin, E; Silva, J. L. Identification of predominant aroma compounds in muscadine grape juice. *J. Food Sci.* **1997**, *62*, 249–252.
5. Baek, H. H.; Cadwallader, K. R. Contribution of free and glycosidically bound volatile compounds to the aroma of muscadine grape juice. *J. Food Sci.* **1999**, *64*, 441–444.
6. Acree, T. E.; Braell, P.; Butts, R. M. The presence of damascenone in cultivars of *Vitis vinifera* (Linneaus), *rotundifolia* (Michaux), and *lambruscana* (Baily). *J. Agric. Food Chem.* **1981**, *29*, 688–690.
7. Shure, K. B.; Acree, T. E. In Vivo and In Vitro Flavor Studies of *Vitis labruscana* Cv., Concord. In *Fruit Flavors: Biogenesis, Characterization, and Authentication*; Rouseff, R. L., Leahy, M. M., Eds.; ACS Symposium Series 596, American Chemical Society: Washington, DC, 1995; pp 127−133.
8. Harkness, E.; Abide, G. Acid Determination for Grape Growers and Winemakers. Proceedings of First Viniculture Short Course, Mississippi

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Publication Date (Web): March 18, 2010 | doi: 10.1021/bk-2010-1035.ch005

Agricultural and Forestry Experiment Station publication, Mississippi State University: Mississippi State, MS, 1986; pp 117−120.

9. Robertson, G. L. The fractional extraction and quantitative determination of pectic substances in grapes and musts. *Am. J. Enol. Vitic.* **1979**, *30*, 182–186.
10. Grosch, W. Detection of potent odorants in food by aroma extract dilution analysis. *Trends Food Sci. Technol.* **1993**, *4*, 68–73.
11. Zhou, Q.; Wintersteen, C. L.; Cadwallader, K. R. Identification and quantification of aroma-active components that contribute to the distinct malty flavor of buckwheat honey. *J. Agric. Food Chem.* **2002**, *50*, 2016–2021.
12. van den Dool, H.; Kratz, P. D. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatograpgy. *J. Chromatogr.* **1963**, *11*, 463–471.
13. Meilgaard, M.; Civille, G. V.; Carr,B. T. *Sensory Evaluation Techniques*, 3rd ed.; CRC Press LLC: Boca Raton, FL, 1999.
14. Baker, R. A. Reassessment of some fruit and vegetable pectin levels. *J. Food Sci.* **1997**, *62*, 225–229.
15. Hartwig, P.; McDaniel, M. R. Flavor characteristics of lactic, malic, citric, and acetic acids at various pH levels. *J. Food Sci.* **1995**, *60*, 384–388.
16. Somogyi, L. P.; Barrett, D. M.; Hui, Y. H. Grape Juice: Factors That Influence Quality, Processing Technology, and Economics. In *Processing Fruits: Science and Technology*; Technomic Pub. Co.: Lancaster, PA, 1996; Vol. 2, p 198.
17. King, R. C.; Sims, C. A.; Moore, L. F.; Bates, R. P. Effects of maturity, skin contact, and carbonation on the quality of sterile-filtered white muscadine grape juice. *J. Food Sci.* **1988**, *53*, 1474–1476, 1485.
18. Acree, T. E. The Odor Quality of Labrusca Grapes. In *Quality of Selected Fruits and Vegetables of North America*; Teranishi R., Barrera-Benitez, H., Eds.; American Chemical Society: Washington, DC, 1981; pp 11−19.
19. Acree, T. E.; Lavin, E. H.; Nishida, R.; Watanabe, S. *o*-Aminoacetophenone, the “Foxy” Smelling Component of Grapes. In *Flavor Science and Technology*; Bessiere, Y., Thomas, A. F., Eds.; John Wiley and Sons: New York, 1990; pp 49−52.
20. Rychlik, M.; Schieberle, P.; Grosch, W. *Compilation of Odor Thresholds, Odor Qualities and Retention Indices of Key Food Odorants*; Deutsche Forschungsanstalt für Lebensmittelchemie and Institut für Lebensmittelchemie der Technischen Universität München: Garching, Germany, 1998.
21. Devos, M.; Patte, F.; Rouault, J.; Laffort, P.; Van Gemert, L. J. *Standardized Human Olfactory Threshold*; Oxford University Press: New York, 1990.
22. Buttery, R. G.; Takeoka, G. R.; Ling, L. C. Furaneol: Odor threshold and importance to tomato aroma. *J. Agric. Food Chem.* **1995**, *43*, 1638–1640.

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Chapter 6

Effect of Edible Coating on Volatile Compounds of Hardy Kiwifruit during Storage

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Hardy kiwifruit (*Actinidia arguta*) is gaining popularity quickly due to its flavor and high nutritional value. The aroma compounds in Hardy kiwifruit (*Actinidia arguta*) were identified using HS-solid phase microextraction and gas chromatogrphy-olfactometry. Based on the odor intensity, the most important aroma compounds in hardy kiwifruit were identified as ethyl butanoate, furaneol, 1-penten-3-one, pentanal, hexanal, (*E*)-2-hexenal, 1-octen-3-ol, linalool, terpinen-4-ol, and α-terpineol. Under ripened hardy kiwifruits were coated with a commercial edible coating material (Semperfresh™), and the aroma compounds were analyzed over 10 weeks of storage. The contents of esters, terpinen-4-ol and α-terpineol increased while aldehydes, myrcene and terpinolene decreased during storage. Both the coated and the control samples followed the same trend. The content of α-terpineol in the under ripe harvested hardy kiwifruits reached that of tree-ripened fruit after 10 weeks of storage. However, the content of ethyl butanoate in both coated and control samples only reached approximately 50% of the level in vine-ripened fruits, whereas terpinen-4-ol only reached 30% of the vine-ripened fruits.

In Flavor and Health Benefits of Small Fruits; Qian, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

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Introduction

Hardy kiwifruit (*Actinidia arguta*) is native to northeastern Asia. It was introduced to Unite States as an ornamental plant. Due to its high vitamin C content and flavor, the fruit has gained popularity quickly. The Pacific Northwest has the largest concentration of commercial acreage of hardy kiwifruit in the world, with an estimated acreage of 120 acres; most of the crop is planted in Oregon. Hardy kiwifruit plants tolerate temperatures as low as -25°F or so, but are sensitive to late spring frosts (*1*).

Compare with fuzzy kiwifruits (*Actinidia chinensis*), hardy kiwifruits are smaller in size (5 to 15 g), have a green, edible skin and have a similar flavor to fuzzy kiwi. It has been reported that hardy kiwifruits have a higher vitamin C content than most citrus fruits, and have a huge potential for both fresh and processed markets. Hardy kiwifruit are not picked vine-ripe, but are picked "green ripe" or physiologically ripe with ripening triggered with ethylene gas prior to shipping. It has been noticed that fruit quality (aroma, flavor) were different when the fruits were picked green ripe compared with vine-ripe (*2*).

The storage life of hardy kiwifruit is only one to two months (*1*). Edible coatings have been evaluated for extending shelf-life of fresh hardy kiwifruit (*3*). Edible coatings have the potential to reduce moisture loss, restrict oxygen uptake, lower respiration, retard ethylene production, seal in flavor volatiles and carry additional functional ingredients (such as antioxidants and antimicrobial agents) that retard discoloration and microbial growth. Some coatings add shine and luster to commodities, thus making them more attractive and appealing to consumers (*3*).

Volatile composition in kiwifruit has been investigated extensively (*4–11*). The main components identified in these reports include pentanal, (*E*)-2-pentenal, 1-penten-3-one, hexanal, (*Z*)- and (*E*)-2-hexenal, (Z)-2-pentenol, hexanol, (*E*)-, and (Z)-2-hexenol, (*Z*)-, and (*E*)-3-hexenol, methyl and ethyl acetate, propanoate, 2-methylpropanoate, butanoate, 2-methylbutanoate, pentanoate, hexanoate, methyl and ethyl benzoate, α-and β-pinenes, δ-3-carene, and 2,5-dimethyl-4-methoxy-3(2*H*)-furanone.

Aroma active compounds in kiwifruit have also been invesitigated using gas chromatography-olfactometry technique (*9*, *12–15*). Acetaldehyde, dimethyl sulfide, 3-penten-2-ol, (*E*)-2-pentenal, 1-penten-3-one, (*E*)-2-hexenal, hexanal, (*E*)-2-hexenol, heptanal, (*E*,*E*)-2,6-nonadienal, 6-methyl-5-hepten-2-one, 1-octen-3-ol, octanol, carvone, ethyl butanoate, methyl benzoate, methyl (2-methylthio)acetate, and α-terpineol have been identified as the most important contributors to the aroma of the kiwifruit. However, neither the volatile composition nor aroma-active compounds in hardy kiwifruit has ever been studied.

Solid-phase microextraction (SPME) is a solvent free, simple, rapid, reproducible and inexpensive technique which was originally developed to extract organic compounds from aqueous solution (*16*, *17*). It has been extensively used in every aspect of analytical chemistry, including extracting and concentrating the trace aroma compounds in fresh fruit and fruit juice (*18–24*).

The objectives of this study were to identify the aroma active compounds in hardy kiwifruit using gas chromatography-mass spectrometry-olfactometry (GC-

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MS-O), and to investigate the changes of volatile compounds in hardy kiwifruit during storage after being treated with edible coating.

Experimental

Chemicals

Methyl heptanoate, nonanal, 1-methyl-4-isopropenyl-1-cyclohexene (limonene), 1,3,3-trimethyl-2-oxabicyclo[2,2,2]octane (eucalyptol), 4-isopropyl-1-methyl-1,4-cyclohexadiene (γ-terpinene), 1-pentanol, 1-methyl-4-isopropyl benzene (*para*-cymene), 1-methyl-4(1-methylethylidene)cyclohexene (terpinolene), 3,7-dimethyl-1,6-octadien-3-ol (linalool), ethyl acetate, methyl butanoate, ethyl butanoate, 3-methylbutyl acetate, ethyl hexanoate, ethyl cyclohexane carboxylate (ethyl benzoate), acetic acid, *trans*-2-hexenal, ethanol, 1-octen-3-ol and 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (furaneol) were purchased from Aldrich Chemical Co. (Milwaukee, WI). 7-Methyl-3-methylene-1,6-octadiene (myrcene), 3-cyclohexene-1-methanol (α-terpineol), butyl acetate were from K&K laboratory Inc. (Plainview, NY). Methanol, malic acid, glucose, fructose, sucrose were from J. T. Baker (Phillipsburg, NJ). Citric acid, quinic acid, pectin, hexanal were from Sigma Chemical Co. (St. Louis, MO). Ethyl octanoate was from Eastman (Rochester, NY). Methyl cyclohexane carboxylate (methyl benzoate) was from Alfa Aesar (Ward hill, MA). 1-Methyl-4(1-methylethyl)1,3-cyclohexadiene (α-terpinene) was from TCI America (Portland, OR). Cyclohexane carbaldehyde (benzaldehyde) was from PolyScience Inc. (Niles, IL).

Hardy Kiwifruit Samples

Hardy kiwifruits of the cultivar *Ananasnaya* were harvested in September 2004 from a commercial hardy kiwifruit vineyard in Sheridan, OR. Fruit were immediately transported to the Value-Added Fruit and Vegetable Products Laboratory at Oregon State University where they were sorted for uniformity of size, color, and absence of visible defects. Samples were stored overnight at room temperature before coating.

Another sample of vine-ripened hardy kiwifruits (23-25°Brix) was picked in October 2004. They were packed in commercial low-vent plastic clamshell containers and immediately frozen at -23°C.

Coating Preparation and Storage

The coating material used in this study was Semperfresh™ (AgriCoat Industries Ltd., Berkshire, England; distributed by Pace International, Seattle, WA), which is a mixture of sucrose esters of fatty acids, sodium carboxymethylcellulose, and mono-diglycerides of fatty acids. A 1% (w/v) coating solution was prepared by diluting 50% Semperfresh™ concentrate with distilled water. Hardy kiwifruit were randomly assigned to the coating treatment or the control (distilled water) treatment. Fruit were hand-dipped into coating solutions for 30 s and dried on stainless steel screens under forced air for 30 min. Samples were then dipped in the coating solution a second time for 30 s and dried to ensure surface dryness (30~ 60 min). Fruits were occasionally rotated to accelerate the drying procedure and to ensure even coating layer formation, taking care to only handle the ends of the fruit. The coated samples were packed in commercial low-vent plastic clamshell containers (8 fruit per pack) under refrigeration conditions (2°C, 88% RH, without light) for up to 10 weeks.

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Sampling and Headspace SPME of Volatile Compounds

Samples were taken after storage of 5, 6, 7, 8, 9, and 10 weeks and were transferred to a frozen room (-23°C) until aroma analysis. The fruit sample (4 fruit) were immerged into liquid nitrogen for 1 min and then were blended to a powder (blending 10 s each time, three times) in a blend jar (Osterizer, Mexico) filled with argon. A portion (8 g) of the fruit powder was quickly transferred to a 20 mL vial which contained a glass stirring bar and a mixture of 0.1 g internal standard solution, 2 g NaCl, 0.1 g $CaCl_2$ and 2 g water. After refilling argon, the vial was tightly closed with a screw-cap with Teflon-coated silicone septum. A SPME fiber (1 cm) coated with 85 μm of Carboxen/Polydimethylsiloxane (Supelco, Bellefonte, PA, USA) was used in this study. Prior to extraction, the fiber was conditioned for 5 min in a GC injection port heated at 250°C. The samples were equilibrated at 35°C for 15 min with constant stirring. The SPME fiber was exposed to the sample headspace at a constant depth for 60 min. The equilibration conditions for temperature and agitation were maintained during extraction of the volatile compounds. Triplicate analysis were performed for each fruit sample. Internal standard solution was not added for the qualitative analysis and GC-Olfactometry analysis.

GC-Olfactometry Analysis

The GC-olfactmetry analysis was performed on a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector (FID) and an olfactometer. The carrier gas was nitrogen at constant pressure (17.5 psi, 1.6 mL/min column flow, measured at 25 °C). The column effluent was split 1:1 (by volume) into the FID (250 °C) and the heated sniffing port. Sample was analysed on a DB-Wax column (30 m × 0.25 mm i.d., 0.5 μm film thickness; J&W Scientific, Folsom, CA) and a XTI-5 column (30 m × 0.25 mm i.d., 0.5 μm film thicknesses; Restek Corporation, Bellefonte, PA) with splitless injection mode. Injection port temperature was 250 °C. The oven temperature was held for 2 min at 40 °C, increased at a rate of 2 °C/min to 100 °C and then 4°C/min to 200°C, finishing with a 3 min hold.

Five panelists (three female and two male) who had more than 50 hours experience of GC-O were selected for this study. They responded to the odor intensity as a 16-point scale ranging from 0 to 15; '0' was none, '7' was moderate, '15' was extreme (*25*). The retention time, aroma intensity value and odor descriptor were recorded. The vine-ripe fruit samples were analysed in duplicate by each panelist. The intensity values were averaged from the ten analyses.

Aroma Compound Identification

The vine-ripe fruit sample was used for volatile compounds identification. GC-MS Analysis was carried out using an Agilent 5973 N GC-MSD system. Both a ZB-wax column (30 m × 0.32 mm i.d., 0.5 μm film thickness; Agilent) and a HP-5 column (30 m × 0.32 mm i.d., 0.5 μm film thickness; Agilent) were used. Helium was used as carrier gas at a constant flow rate of 2 mL/min. Injection port temperature was 250 °C. The oven temperature was held for 2 min at 40 °C, increased at a rate of 2 °C/min to 100 °C and then 4°C/min to 200°C, finishing with a 3 min hold. The electron impact (EI) energy was 70 eV, and the ion source temperature was set at 230 °C. System control and data analysis were performed with Enhanced ChemStation Software, GCA v. C.00.01.08 (Agilent Technologies Inc.). Compounds were tentatively identified by comparing their mass spectra with those in the Wiley 275.L (G1035) Database (Agilent Technologies Inc.), and then confirmed by their retention indices (RI) and odor descriptors. Retention indices were estimated in accordance with a modified Kováts method (*26*).

Aroma Compounds Quantification

A synthetic matrix made up of 5 g/L of malic acid, 10 g/L of citric acid, 9.6 g/L of quinic acid, 43.2 g/L of glucose, 46 g/L of fructose, 2 g/L of sucrose, 7.4 g/L of pectin was prepared in 1 L volatile-free distilled water (boiled for 30 min). A standard stock solution was prepared in methanol containing 5 g/kg each of ethyl acetate, methyl butanoate, ethyl butanoate, butyl acetate, hexanal, 3-methylbutyl acetate, myrcene, *α*-terpinene, limonene, eucalyptol, *trans*-2-hexenal, ethyl hexanoate, *γ*-terpinene, *p*-cymene, terpinolene, ethyl octanoate, 1-octen-3-ol, benzaldehyde, linalool, methyl benzoate, ethyl benzoate and *α*-terpineol. Seven standard working solutions were obtained by diluting standard stock solution with methanol to final concentrations 6.25, 12.5, 25, 50, 100, 200 and 400 mg/kg. The internal standard stock solution was prepared by dissolving methyl heptanoate and nonanal in methanol at concentration of 5 g/kg, and diluted to 50 mg/kg as the internal standard work solution. Methyl heptanoate was used as internal standard to construct standard curves for ethyl acetate, methyl butanoate, ethyl butanoate, butyl acetate, 3-methylbutyl acetate, myrcene, *α*-terpinene, limonene, eucalyptol, ethyl hexanoate, *γ*-terpinene, *p*-cymene, terpinolene, ethyl octanoate, linalool, methyl benzoate, ethyl benzoate and *α*-terpineol, and nonanal was used for 1-octen-3-ol, hexanal, *trans*-2-hexenal and benzaldehyde.

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Table 1. Aroma Components in Vine-Ripe Hardy Kiwifruit

Compounds	*RI on*		*Basis of identification*	*Descriptor*	*Osme value*	
	ZB-wax	*HP-5*			*ZB-wax*	*HP-5*
ethyl acetate	896	619	MS, RI, odor	fruity	1	1
pentanal	976	698	MS, RIL, odor	green, earthy, fatly	5	4
methyl butanoate	985	719	MS, RI, odor	fruity, sweet	1	1
1-penten-3-one	1013	678	MS, RIL, odor	cut grass, chemical	6	5
ethyl butanoate	1033	801	MS, RI, odor	fruity, sweet	10	10
butyl acetate	1069	813	MS, RI, odor	fruity, juicy	3	2
hexanal	1079	799	MS, RI, odor	cut grass	4	4
3-methylbutyl acetate	1112	874	MS, RI, odor	banana	2	2
trans-2-pentenal	1125	748	MS, RIL, odor	fruity, sweet	1	1
α-terpinene	1170	1011	MS, RI, odor	fruity	1	1
eucalyptol	1196	1025	MS, RI, odor	sweet, clove	1	1
trans-2-hexenal	1214	846	MS, RI, odor	green, fruity	5	5
ethyl hexanoate	1232	1000	MS, RI, odor	fruit	2	1
pentanol	1249	761	MS, RI, odor	tropical fruit	4	3
terpinolene	1274	1082	MS, RI, odor	floral, pine,	1	1
ethyl octanoate	1427	1195	MS, RI, odor	fruity, floral	1	0
1-octen-3-ol	1440	976	MS, RI, odor	mushroom	5	5
acetic acid	1451	nd	MS, RI,	vinegar	1	0
benzaldehyde	1493	951	MS, RI, odor	almond, sweet	2	1
linalool	1543	1096	MS, RI, odor	floral,sweet	4	3
terpinen-4-ol	1593	1170	MS, RIL, odor	pine, floral	4	3
methyl benzoate	1612	1089	MS, RI, odor	fruity, sweet	4	4
ethyl benzoate	1659	1165	MS, RI, odor	floral, celery	4	4

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Table 1. (Continued). Aroma Components in Vine-Ripe Hardy Kiwifruit

Compounds	*RI on*		*Basis of identification*	*Descriptor*	*Osme value*	
	ZB-wax	*HP-5*			*ZB-wax*	*HP-5*
α-terpineol	1692	1184	MS, RI, odor	mint, green tea	3	2
furaneol	2053	1059	RI, odor	caramel, sweet	9	9

An aliquot (0.1 g) of each standard work solution and an aliquot (0.1 g) internal standard work solution were mixed with 9.8 g synthetic matrix to give 6 levels of stardard solutions. The final concentrations of the standard compounds were 0.0625, 0.125, 0.25, 0.5, 1, 2, 4 mg/kg, respectively, and the internal standard concentrations were 0.5 mg/kg. These samples were extracted by using the SPME technique described previously. Calibration curves were constructed based on applying linear regression analysis on the concentration (mg/kg compound / mg/kg internal standard) and peak area (area of compound / area internal standard) ratios. A DB-Wax column (30 m × 0.25 mm i.d., 0.5 μm film thickness; J&W Scientific) was used for the quantitative analysis. The GC conditions were the same as described previous except the column splitter was not used. Chromatographic data were processed through a PeakSimple software v. 2.83 (SRI Instruments, Torrance, CA).

The volatile composition in hardy kiwifruit was analyzed based on the respective calibration curve of standard in synthetic juice. Terpinen-4-ol was estimated with standard curve of α-terpineol, pentanal was estimated with hexanal, 1-penten-3-one and *trans*-2-pentenal were estimated with *trans*-2-hexenal.

Results and Discussion

Preparation of Samples and Extraction of the Volatiles

The technique used for sample preparation is extremely important for both qualitative and quantitative analysis of aroma in food system. It is particularly important for analysis of fruit and vegetable aroma as the fruit and vegetable enzyme systems can be quickly activated after the cells are broken, resulting the formation of new aroma compounds. The rapid formation of aldehydes by lipoxygenase system in fruits and vegetables upon cellular disrupture is one example. To minimize the effect of system enzymes on the volatile profile of fruits and vegetables, many measures have been taken including preparing samples in low temperature (*27*), addition of salts such as $SnCl_2$, NaCl, $CaCl_2$, $CuSO_4$ (*5*, *28–30*) to inhibit the activation of enzymes.

In the present study, vine-ripe hardy kiwifruit was used to optimize the parameters of sample preparation and SPME extraction. Temperature of blending, SPME extraction temperature, time and sample amount, dosages of NaCl, $CaCl_2$ and atmosphere of headspace were examined. Ultra-low temperature homogenization (blending with liquid nitrogen) was used to produce puree powder. This operation not only benefits deactivating enzyme systems but also is easy to transfer fruit puree. Saturated $CaCl_2$ was selected to inhibit enzyme systems during SPME extraction. In addition, argon was used to exclude oxygen on the headspace. By extracting the volatiles as described in the experimental method, the concentrations of pentanal, *trans*-2-pentenal, hexanal and *trans*-2-hexenal were 37.3%, 20.3%, 24.2% and 31.8% lower, respectively, than that produced by blending at 25°C and extracting without added $CaCl_2$, while changes of other analytes were not more than ±4.7 %.

By comparing the total peak area of 32 volatiles and the fruit aroma characteristics after SPME extraction, it was determined that the volatiles in hardy kiwifruit was be best extracted at 35°C for 50 min.

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Table 2. Volatile Concentration of Vine-Ripe Hardy Kiwifruit

Compound	*Regression equation*	*R^2*	*Conc. (mg/kg)*	*Odor threshold (mg/kg) (36)*
methyl heptanoate (IS)				
ethyl acetate	y=0.049x	0.981	5.02±0.42	10
methyl butanoate	y=0.122x	0.993	0.42±0.02	0.06
ethyl butanoate	y=0.101x	0.964	13.04±0.69	0.001
butyl acetate	y=0.123x	0.995	0.17±0.01	0.006
3-methylbutyl acetate	y=0.128x	0.999	0.03±0.005	0.006
myrcene	y=0.882x	0.980	0.17±0.01	0.036
α-terpinene	y=0.155x	0.991	0.02±0.003	0.085
limonene	y=1.758x	0.990	0.04±0.005	1
eucalyptol	y=0.117x	0.998	0.16±0.03	0.023
ethyl hexanoate	y=0.676x	0.999	<0.01	0.001
γ-terpinene	y=1.771x	0.993	0.02±0.002	1
p-cymene	y=1.606x	0.983	0.04±0.005	0.1
terpinolene	y=2.059x	0.994	0.11±0.01	0.2
ethyl octanoate	y=6.025x	0.997	<0.01	0.2
linalool	y=0.082x	0.975	0.14±0.02	0.006
methyl benzoate	y=0.900x	0.955	0.04±0.004	0.11
ethyl benzoate	y=1.000x	0.962	0.11±0.01	0.06
α-terpineol	y=0.039x	0.938	1.27±0.11	0.086
terpinen-4-ol			1.29±0.10	0.34

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Table 2. (Continued). Volatile Concentration of Vine-Ripe Hardy Kiwifruit

Compound	*Regression equation*	*R^2*	*Conc. (mg/kg)*	*Odor threshold (mg/kg) (36)*
Nonanal (IS)				
1-octen-3-ol	y=0.038x	0.982	1.18±0.18	0.01
pentanal			1.28±0.32	0.11
hexanal	y=0.037x	0.973	1.62±0.32	0.009
trans-2-pentenal			0.19±0.06	0.24
trans-2-hexenal	y=0.107x	0.998	0.43±0.07	0.082
1-penten-3-one			0.13±0.01	0.00073
benzaldehyde	y=0.195x	0.980	0.09±0.006	1

Aroma-Active Compounds and Volatile Composition in Hardy Kiwifruit

The aroma-active volatile compounds in vine-ripe hardy kiwifruit were identified on both HP-5 and ZB-Wax columns. The identifications were achieved through mass spectral matching and verified by comparison with Kovats RI (or RIL) and odor descriptors (Table 1). Although the aroma composition of hardy kiwifruit has never been studied, many of the volatile compounds have been identified in fuzzy kiwifruit (*Actinidia chinensis*) by previous works (*9*, *12–15*). In this study, a few more terpenoids, esters, and 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (furaneol) were identified in hardy kiwifruits.

Terpenoids could make some contribution to hardy kiwifruit aroma. Linalool, terpinen-4-ol and α-terpineol had medium odor intensity (intensity = 2-4) and contribute flower, pine and mint odorant respectively. α-Terpinene and terpinolene could just be detected at sniffing port with fruity, floral and pine odor. Eucalyptol gave sweet and clove-like aroma with low intensity. Sabinene, myrcene, limonene, γ-terpinene, *para*-cymene were also identified in the hardy kiwifruit on both columns, but they did not contribute any aroma. Although each terpenoid compound had relatively low aroma intensity, but they can act synergistically, and could present floral, pine, and mint aroma to hardy kiwifruit flavor profile.

Among nine esters identified in hardy kiwifruit, ethyl esters dominated the profile. Ethyl butanoate had a high aroma intensity value (=10), followed by methyl benzoate and ethyl benzoate (aroma intensity value = 4). All of these esters gave fruity, floral and sweet aroma.

Pentanal, hexanal and *trans*-2-hexenal had slight to moderate odor intensity (aroma intensity value = 4-5), *trans*-2-pentenal and benzaldehyde could just be detected.

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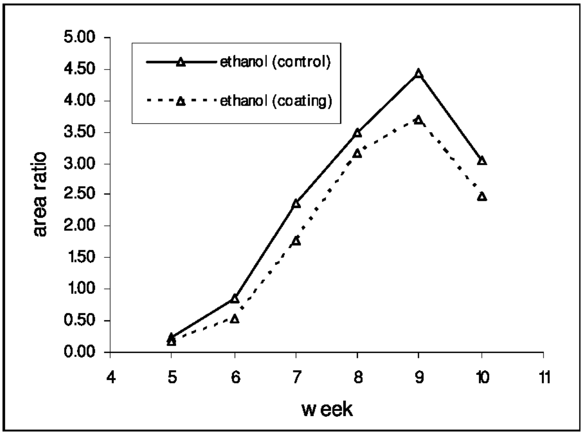

Figure 1. Effect of "Semperfresh™" edible coating on ethanol in hardy kiwifruit during storage

2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone (furaneol) was tentatively identified by its RI and descriptive odor property. It had characteristic caramel, sweet and cotton candy-like odor and much lower aroma threshold. Based on the odor intensity (Intensity value = 9), furaneol seem to play an important role in hardy kiwifruit aroma. Although it has not been previously identified in kiwifruit, this compound has been identified as a very important aroma compound in many fruits such as pineapple, strawberry, raspberry, tomato and many other fruits (*31–34*).

Acetic acid was only acid identified on ZB-wax column. However, because its odor threshold is quite high (~24 ppm), its contribution to fruit aroma may be limited. 1-Octen-3-ol gave a typical mushroom odor at the end of both columns.

Selected aroma-active compounds and terpenes in vine-ripened hardy kiwifruits were qualified using HS-SPME-GC-FID. Standard curves for twenty-two interesting aroma compounds were constructed. The linear correlation coefficients varied (R^2 0.938-0.999) depending on the compound (Table 2). Two internal standards were used for more accurate quantification (*35*). Ethyl butanoate had the highest concentration followed by ethyl acetate. Ethyl butanoate has a very low odor threshold of 0.001 mg/kg in water (*36*), and its aroma contribution to vine-ripened hardy kiwifruit should be significant. Although having low concentrations, methyl butanoate, butyl acetate and 3-methylbutyl acetate should all contribute to the aroma due to their even lower aroma thresholds. Pentanal, hexanal, 1-octen-3-ol, terpinen-4-ol and α-terpineol all had concentrations greater than 1 mg/kg, all of them have low odor thresholds, thus could be important aroma contributors. Except for myrcene, all terpenes had concentrations lower than their odor thresholds. Furaneol was not quantified because the HS-SPME method is not sensitive for this compound.

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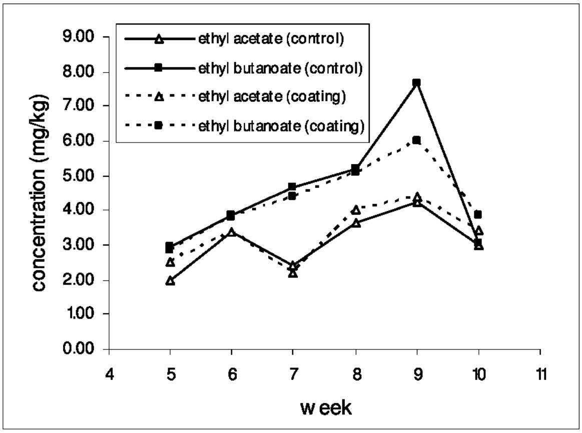

Figure 2. Effect of "Semperfresh™" edible coating on ethyl acetate and ethyl butanoate in hardy kiwifruit during storage

Changes of Aroma Compounds in Hardy Kiwifruit during Storage

Volatile compostion in fruits is highly dependent on ripening status and many other factors. Post-harvest storage can be particularly important for flavor development and deterioration. Gas composition, packaging, storage temperature and time all can affect the volatile composition of the fruits. Edible films and coatings from biopolymers have been widely used on fresh fruits and vegetables as oxygen and water vapor barriers to extend shelf lives. "Semperfresh" is a mixture of esters of mono- and di-glycerides with sucrose and carboxymethylcellulose. When applied to the surface of the fruits, it reduces fruit gas exchange and water loss. As shown in Figure 1, ethanol content increased during storage up to 9 weeks and then decreased at 10 weeks. The increase in ethanol concentration was consistent with the metabolism law of fruit storage. The changing trend during storage in control and coated hardy kiwifruit were very similar suggesting the coating did not change the ethanol metabolism.

The concentration of esters, which were represented by ethyl acetate and ethyl butanoate, increased from the 5th week to the 9th week (Figure 2), which may contribute to a more fruity and sweet aroma note. However, their concentration decreased quickly from the 9th week to the 10th week, which was related to dehydration of fruit. The concentration of ethyl butanoate even at its peak formation was still only 54% of vine-ripened fruit. The observation of ester accumulation during storage was consistent with earlier reports (*5*, *18*, *37*). "Semperfresh" edible coating had no effect on the formation of ethyl acetate and ethyl butanoate.

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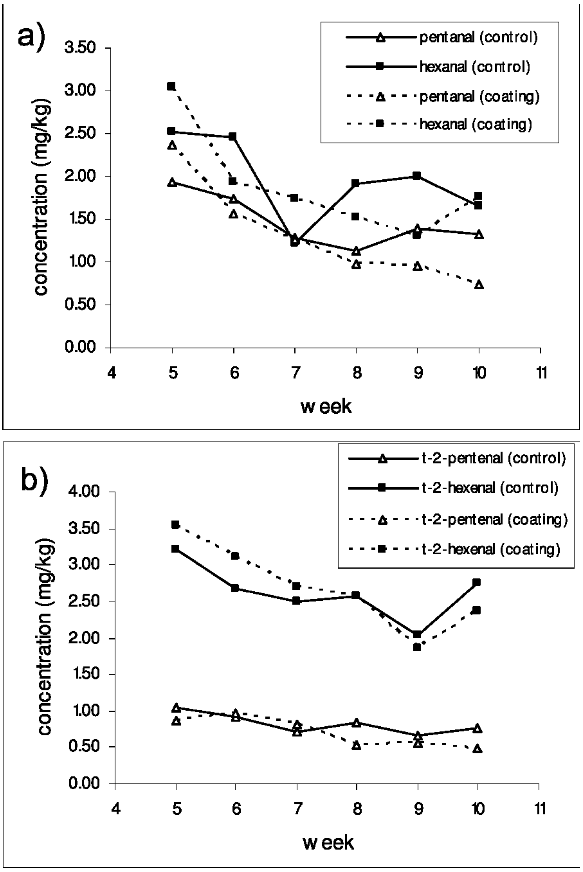

Figure 3. (a) Effect of "Semperfresh™" edible coating on pentanal and hexanal in hardy kiwifruit during storage; (b) Effect of "Semperfresh™" edible coating on trans-2-pentenal and trans-2-hexenal in hardy kiwifruit during storage

During storage, the levels of four alkyl and alkenyl aldehydes decreased (Figure 3a, 3b) for both the control and coated hardy kiwifruit. This result was different from the work on kiwifruit during storage reported by Wan *et al.* (*18*) but similar to studies between different ripening stages of kiwifruit reported by Pfannhauser and Young et al. (*6*, *37*). The reduction of C-6 compounds accompanied by an increase in ethyl butanoate was correlated with aroma impression shift from a fresh-green to a more ester-like note (*6*).

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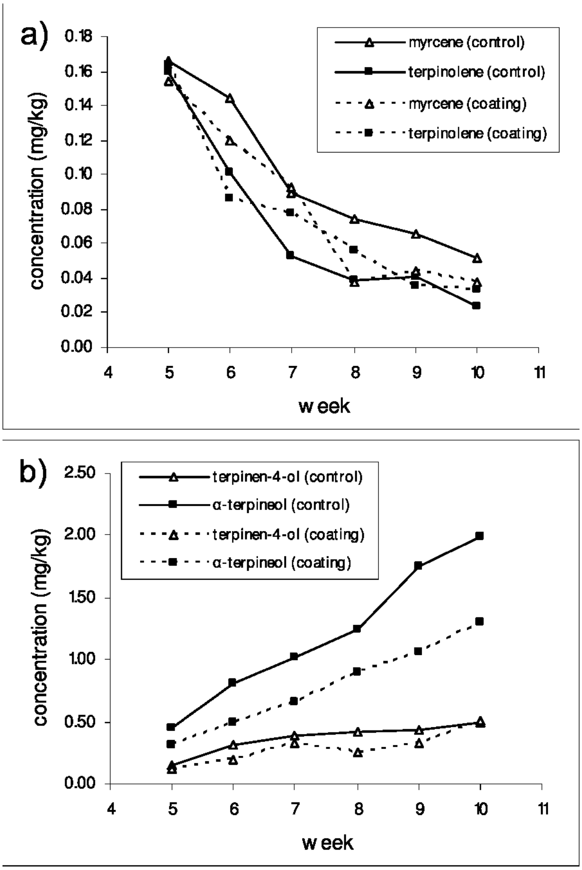

Figure 4. (a) Effect of "Semperfresh™" edible coating on myrcene and terpinolene in hardy kiwifruit during storage; (b) Effect of "Semperfresh™" edible coating on terpinen-4-ol and α-terpineol in hardy kiwifruit during storage

It is interesting to observe that all terpenes, particularly myrcene and terpinolene, decreased during storage for both the control and "Semperfresh™" coated hardy kiwitfruit (Figure 4a). There was no difference in the degree of decrease between the control and the "Superfresh" treatment. Terpene alcohols, however, increased during storage (Figure 4b). Terpinen-4-ol only increased slightly during the ten weeks of storage and "Semperfresh™" treatment did not change its accumulation. A 4-fold increase during storage was observed for α-terpineol for the control treatment. "Semperfresh™" coating retarded the accumulation of α-terpineol in hardy kiwifruit. Since aroma thresholds of most of the terpene alcohols were very low, the differences in terpene alcohol accumulation may change the aroma perception of hardy kiwifruit after storage.

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References

1. Strik, B.; Hummer, K. 'Ananasnaya' hardy kiwifruit. *J. Am. Pomological Soc.* **2006**, *60* (3), 106–112.
2. Fisk, C. L.; McDaniel, M. R.; Strik, B. C.; Zhao, Y. Physicochemical, sensory, and nutritive qualities of hardy kiwifruit (*Actinidia arguta* 'Ananasnaya') as affected by harvest maturity and storage. *J. Food Sci.* **2006**, *71* (3), S204–S210.
3. Fisk, C. L.; Silver, A. M.; Strik, B. C.; Zhao, Y. Postharvest quality of hardy kiwifruit (*Actinidia arguta* 'Ananasnaya') associated with packaging and storage conditions. *Postharvest Biol. Technol.* **2008**, *47* (3), 338–345.
4. Takeoka, G. R.; Guntert, M.; Flath, R. A.; Wurz, R. E.; Jennings, W. Volatile constituents of kiwi fruit (*Actinidia chinensis* Planch.). *J. Agric. Food Chem.* **1986**, *34* (3), 576–578.
5. Bartley, J. P.; Schwede, A. M. Production of volatile compounds in ripening kiwi fruit (*Actinidia chinensis*). *J. Agric. Food Chem.* **1989**, *37* (4), 1023–1025.
6. Pfannhauser, W. Sensorial and instrumetnal analytical investigations of kiwi fruit flavor. *Z. Lebensm.-Unters. Forsch.* **1988**, *187* (3), 224–228.
7. Pfannhauser, W.; Kellner, R.; Fischboek, G. GC/FTIR and GC/MS analysis of kiwi flavors. *Dev. Food Sci.* **1990**, *24*, 357–373 (Flavors Off-Flavors '89).
8. Pino, J. A. Volatile constituents of tropical fruits. IV. Kiwi, carambola, and mangosteen. *Alimentaria (Madrid)* **1997**, *286*, 47–50.
9. Jordan, M. J.; Margaria, C. A.; Shaw, P. E.; Goodner, K. L. Aroma active components in aqueous kiwi fruit essence and kiwi fruit puree by GC-MS and multidimensional GC/GC-O. *J. Agric. Food Chem.* **2002**, *50* (19), 5386–5390.
10. Moio, L.; Di Marzio, L.; Genovese, A.; Piombino, P.; Squillante, E.; Castellano, L.; Mercurio, V. Sensory descriptors and volatile components in enhanced olfactory impact of Fiano di Avellino wine aroma. *Vignevini* **2002**, *29* (4), 115–123.
11. Winterhalter, P. Fruits [tropical, aroma compounds]. IV. *Food Sci. Technol. (New York, NY, United States)* **1991**, *44*, 389–409 (Volatile Compd. Foods Beverages).
12. Young, H.; Paterson, V. J.; Burns, D. J. W. Volatile aroma constituents of kiwifruit. *J. Sci. Food Agric.* **1983**, *34* (1), 81–85.
13. Frank, D.; O'Riordan, P.; Varelis, P.; Zabaras, D.; Watkins, P.; Ceccato, C.; Wijesundera, C. Deconstruction and recreation of 'Hayward' volatile flavour using a trained sensory panel, olfactometry and a kiwifruit model matrix. *Acta Hortic.* **2007**, *753*, 107–118 (Proceedings of the VI International Symposium on Kiwifruit, 2006, Volume 1).
14. Friel, E. N.; Wang, M.; Taylor, A. J.; MacRae, E. A. In Vitro and in vivo release of aroma compounds from yellow-fleshed kiwifruit. *J. Agric. Food Chem.* **2007**, *55* (16), 6664–6673.
15. Young, H.; Perera, C. O.; Paterson, V. J. Identification of E-hex-3-enal as an important contributor to the off-flavor aroma in kiwifruit juice. *J. Sci. Food Agric.* **1992**, *58* (4), 519–522.

16. McDonnell, T.; Pawliszyn, J. Solid phase microextraction with thermal desorption fused silica optical fibres. *J. Anal. Chem.* **1990**, *62*, 2145–2148.
17. Arthur, C. L.; Killam, L. M.; Motlagh, S.; Lim, M.; Potter, D. W.; Pawliszyn, J. Analysis of substituted benzene compounds in groundwater using solid-phase microextraction. *Environ. Sci. Technol.* **1992**, *26* (5), 979–83.
18. Wan, X. M.; Stevenson, R. J.; Chen, X. D.; Melton, L. D. Application of headspace solid-phase microextraction to volatile flavour profile development during storage and ripening of kiwifruit. *Food Res. Int.* **1999**, *32* (3), 175–183.
19. Jetti, R. R.; Yang, E.; Kurnianta, A.; Finn, C.; Qian, M. C. Quantification of selected aroma-active compounds in strawberries by headspace solid-phase microextraction gas chromatography and correlation with sensory descriptive analysis. *J. Food Sci.* **2007**, *72*, S487–S495.
20. Yang, X.; Peppard, T. L. Solid-phase microextraction for flavor analysis. *J. Agric. Food Chem.* **1994**, *42*, 1925–1930.
21. Song, J.; Gardner, B. D.; Holland, J. F.; Beaudry, R. M. Rapid analysis of volatile flavor compounds in apple fruit using SPME and GC/time-of-flight mass spectrometry. *J. Agric. Food Chem.* **1997**, *45*, 1801–1807.
22. Jia, M.; Zhang, Q. H.; Min, D. B. Optimization of solid-phase microextraction analysis for headspace flavor compounds of orange juice. *J. Agric. Food Chem* **1998**, *46*, 2744–2747.
23. Liu, T.-T.; Yang, T.-S. Optimization of solid-phase microextraction analysis for studying change of headspace flavor compounds of banana during ripening. *J. Agric. Food Chem.* **2002**, *50*, 653–657.
24. Reid, L. M.; O'Donnell, C. P.; Downey, G. Potential of SPME-GC and chemometrics to detect adulteration of soft fruit purées. *J. Agric. Food Chem.* **2004**, *52*, 421–427.
25. Miranda-Lopez, R.; Libbey, L. M.; Watson, B. T.; McDaniel, M. R. Odor analysis of Pinot noir wines from grapes of different maturities by a gas chromatography-olfactometry technique (Osme). *J. Food Sci.* **1992**, *57*, 985–993, 1019.
26. Van den Dool, H.; Kratz, P. A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *J. Chromatogr.* **1963**, *11* (4), 463–471.
27. Buttery, R. G.; Teranishi, R.; Ling, L. C. Fresh tomato aroma volatiles: A quantitative study. *J. Agric. Food Chem.* **1987**, *35* (4), 540–544.
28. Schwimmer, S. *Source Book of Food Enzymology*; Avi: Westport, CT, 1981.
29. Geduspan, H. S.; Peng, A. C. Changes in cucumber volatile compounds on chilling temperature and calcium chloride treatment. *J. Food Sci.* **1986**, *51*, 852–853.
30. Schreier, P.; Lorenz, G. *Flavor '81*; Walter de Gruyter: Berlin, 1981; p 495.
31. Buttery, R. G.; Takeoka, G. R.; Krammer, G. E.; Ling, L. C. Identification of 2,5-dimethyl-4-hydroxy-3(2H)-furanone (furaneol) and 5-methyl-4-hydroxy-3(2H)-furanone in fresh and processed tomato. *Food Sci. Technol. (London)* **1994**, *27* (6), 592–594.

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32. Rodin, J. O.; Himel, C. M.; Silverstein, R. M.; Leeper, R. W.; Gortner, W. A. Volatile flavor and aroma components of pineapple. I. Isolation and tentative identification of 2,5-dimethyl-4-hydroxy-2,3-dihydro-3-furanone. *J. Food Sci.* **1965**, *30*, 280–285.
33. Willhalm, B.; Stoll, M.; Thomas, A. F. 2,5-Dimethyl-4-hydroxy-2,3-dihydrofuran-3-one. *Chem. Ind. (London, U. K.)* **1965**, *28*, 1629–1630.
34. Honkanen, E.; Pyysalo, T.; Hirvi, T. The aroma of Finnish wild raspberries, Rubus idaeus, L. *Zeitschrift fuer Lebensmittel-Untersuchung und -Forschung* **1980**, *171* (3), 180–182.
35. Qian, M.; Reineccius, G. Static headspace and aroma extract dilution analysis of Parmigiano Reggiano cheese. *J. Food Sci.* **2003**, *68* (3), 0794–0798.
36. Gemert, L. J. v. *Compilations of Odour Threshold Values in Air, Water and Other Media*; Boelens Aroma Chemical Information Service: Huizen, The Netherlands, 1999.
37. Young, H.; Paterson, V. J. The effects of harvest maturity, ripeness and storage on kiwifruit aroma. *J. Sci. Food Agric.* **1985**, *36* (5), 352–358.

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Chapter 7

Effects of Germplasm Origin and Fruit Character on Volatile Composition of Peaches and Nectarines

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Ninety-five volatile chemicals in peaches and nectarines with different fruit characters representing different germplasm origins were investigated using HS-SPME-GC-MS. The result showed that the composition and content of volatiles depended on germplasm origins and fruit characters. Chinese bred cultivars obtained from Chinese local and foreign cultivars and Japanese cultivars had significantly higher contents of total volatiles and esters than Chinese local cultivars. White-flesh flat peaches had the highest contents of total volatiles and esters, which were significantly higher than those of other groups with different fruit characters. Terpenoids in white-flesh flat peaches was significantly higher than that in white-flesh nectarines. Ten cultivars including 'Yutian', 'Ruipan 3', 'Yangzhou 124

In Flavor and Health Benefits of Small Fruits; Qian, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

Pantao', 'Beijing 5', 'Fenglu', 'Kanto 5', 'Okitsu', 'Charme', 'Babygold 5' and 'Babygold 6' with high contents of lactones, terpenoids and total volatiles are considered the desirable cultivars that can be used for further breeding.

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Introduction

It is well known that peaches and nectarines are native to China where peaches have been cultivated for at least 3000 years, and nectarines for over 2000 years. Peach tree was introduced into Europe at the beginning of the Roman era and then into the United States during the 19th century. With a long history of cultivation and extensive geographical distribution, there are a rich germplasm resources of peaches and nectarines in China and all over the world. However, regardless of the significant development of novel cultivars and increase of fruit production, many peaches and nectarines are often criticized about their undesirable qualities, which were affected by size, external color, texture (e.g., firmness), and contents of sugars, acids, as well as aromas.

As one of the essential indices of fruit quality, aroma-impact flavors of peaches and nectarines have been intensively studied, resulting in the identification of more than 110 volatile compounds (*1–11*), which include C_6 compounds, alcohols, aldehydes, esters, terpenoids, ketones and lactones. Among the lactones, particularly γ- and δ-decalactones, have been reported to be the major contributors to the peach aroma, while other volatiles such as C_6 aldehydes, alcohols and terpenoids, also made a certain degree of contribution (*12–15*).

Moreover, intensive investigations have focused on the aroma evolution of peach and nectarine during ripening (*3*, *7*, *8*, *12*, *15*, *16*) and cold storage (*9*, *17*). It is well known that the composition and content of volatile aromas have significant changes during the maturation. For example, in immature fruits, C_6 compounds are the initial major aroma contributors, but their levels decrease drastically while those of lactones, benzaldehyde and linalool increase significantly during maturation (*5*, *12*). Several studies have also investigated the effect of culturing techniques and managements on composition and content of volatiles, which could be changed by orchard management, such as fertilization (*18*), storage (*19*), climate or microclimate conditions such as sun light (*20*), and postharvest treatment (*4*, *7*, *21*, *22*). Volatile composition is also cultivar dependent. Engel et al. concluded that nectarines contained significantly higher amounts of δ-decalactone than peaches (*5*, *13*). Robertson et al. reported that white-flesh peaches contained more linalool than yellow-flesh cultivars (*23*).

Headspace solid-phase microextraction (HS-SPME) was a sampling technique based on sorption of analytes on a polymeric material that is coated on a silica fiber. It has been widely used for the analyses of volatile and semi-volatile compounds of food and fruits (*11*, *24–30*) since its introduction by Zhang and Pawliszyn in 1993 (*31*). The HS-SPME technique has the following advantages: low cost, small amount of sample required, no solvent contamination, short time of extraction, simplicity, high selectivity and sensibility (*32*). The HP-SPME method involves two steps: the partitioning of the analytes between the coating material and the sample; and the thermal desorption of the analytes into gas chromatograph (*28*).

In this study, HP-SPME combined with gas chromatography-mass spectrometry (GC-MS) was applied to study the characteristic volatiles of peaches and nectarines at the germplasm level. This study mainly focused on comparing the aroma composition and content among the germplasm resources, which include Chinese local cultivars and a number of cultivars from other countries in order to acquire information for future breeding efforts aiming at enhancing fruit quality via effects on aroma.

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Materials and Methods

Plant Materials

A total of 95 peach (*Prunus persica* L. Batsch) cultivars were used to study the aroma composition and content (Table 1). All the samples were collected from the Germplasm Repository for peach in the Institute of Forestry and Pomology, Beijing Academy of Agriculture and Forestry Science, in 2007. The trees, grafted on a wild *P. persica* rootstock, were planted in 3 m apart within rows and 5 m apart between rows in the spring of 1996. They were trained to ‘Y’ training systems and pruned by the long pruning method in winter (*33*). The same orchard managements, such as fertilization and irrigation were applied in the whole orchard. The fruits were picked from three trees of each cultivar when the green color of the fruit skin has almost disappeared. At the same time, the ground color of white-fleshed peaches turned milk white, whilst the ground color of yellow-fleshed peaches turned yellow or orange. All the fruits were picked from the southern or western crown about 1.5-2 m high from the ground of the tree at maturity. The fruit samples were taken to the laboratory immediately after harvest, washed by deionized water and surface-dried with gauze. Then three slices were taken from different orientations of each fruit. Three fruits were used for one composite sample per tree, and considered as one replication resulting in three replications for every cultivar. Samples were ground to a powder in liquid nitrogen and stored at -40°C until analysis.

Isolation and Concentration of Volatiles

For headspace sampling, SPME fibers coated with poly-dimethylsiloxane-divinylbenzene (65 μm, PDMS/DVB) (Supelco Co., Bellefonte, PA, USA) were used by optimization of the method carried out in a previous work (*24*). The fiber was activated according to the manufacturer’s instructions. The SPME method was used for the isolation and concentration of volatiles. For each extraction, 2 g of the pulp powder, 40 μL 3-octanol (0.814 mg L^{-1}, added as an internal standard), 0.6 g NaCl and 0.4 g $CaCl_2$ (added to increase partitioning of volatiles from the

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liquid into the gas phase and to restrain the enzyme activity, respectively) (*34*, *35*) were placed in a 4 mL capped vial. The vial was placed in a 45°C water bath with a consistent magnetic stirring when the SPME fiber was exposed to the headspace of the sample to adsorb the volatile analytes for 30 min. The fiber was then introduced into the GC injector port for desorption at 220°C for 2 min in the splitless mode. Linear retention index (LRI) were calculated by using *n*-alkane standards (C_5-C_{40}) based on the method of Ceva-Antunes, et al. (*36*).

Table 1. Peach and nectarine cultivars are listed according to their origins. The number in parenthesis following the cultivar indicates the accession number.

Populations	*Characters*	*Taxa*
	WF[b]	Baimangpantao (1)
	WP	Baifentao (2), Baiyintao (3), Cuixiangtao (4), Jinhongtao (5), Linbai 4 (6), Qingmaozibaihuatao (7), Xiaobaihua (8)
C_l[a]	YP	Huanglu (9), Zaoshenghuangjin (10)
	WF	Ruipan 4 (11), Ruipan 5 (12)
	WP	Baihuatao (13), Ji 2102 (14), Lvhua 7 (15), Qiuxiangmi (16), Wanshuomi (17), Xinbaihua (18), Yulu (19), Yutian (20), Zaobaimi (21)
Cb	YP	Chengyan (22), Jinshiji (23), Wanshiji (24)
	WF	Ruipan 3 (25), Yangzhou124 Pantao (26)
	WP	Beijing 5 (27), Fenglu (28), Huayu (29), Jingyu (30), Qinxuan 2 (31), Wanhongmi (32), Xiahui 6 (33), Yihongshuimi (34), Zaoyu (35)
	YP	Guihuang (36), Qiukui (37), Yanfeng (38)
	WN	Ruiguang 2 (39), Ruiguang 7 (40), Ruiguang 27 (41)
CF	YN	81-26-4 (42), 90-8-18 (43), 94-6-19 (44)
	WP	Hakuto (45), Nippon Suimitsu (46), Okayama 500 (47), Shin Musume (48), To Hakuho (49)
	YP	Ban Ougon (50), Kanto 5 (51), Myojo (52)
J	YN	Okitsu (53)
	WP	Charme (54), Chimarrita (55), Corol (56), Fuzalode (57), J7 (58), NJ250 (59), NJ257 (60), P6 (61)

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Table 1. (Continued). Peach and nectarine cultivars are listed according to their origins. The number in parenthesis following the cultivar indicates the accession number.

Populations	*Characters*	*Taxa*
	YP	Androreda (62), Babygold 5 (63), Babygold 6 (64), Babygold 9 (65), Bulgaria 2(66), Caullinan (67), Dixon (68), Early Crawford (69), Emilia (70), Fortuna (71), Fuzador (72), Glohaven (73), Harbrita (74), Havis (75), Loring (76), Mcneely (77), NJC47 (78), NJC77 (79), NJC237 (80), Norman (81), Redtop (82), Riogrand (83), Shasta (84), Vivian (85)
AE	YN	Flavortop (86), French Nectarine (87), Great Diamond (88), Legrand (89), NJN78 (90), Nectared 4 (91), Nectared 6 (92), P1 (93), Tasiva (94), Vega (95)

[a] C_l Chinese local cultivars; C_b pure Chinese original bred cultivars; CF China × foreign cultivars; J Japanese cultivars; AE American and European cultivars [b] WF= white-flesh flat peaches; WP= white-flesh peaches; YP= yellow-flesh peaches; WN= white-flesh nectarines; YN= yellow-flesh nectarines

GC-MS Conditions

The volatile constituents were analyzed by an Agilent (Palo Alto, CA) 5975 mass selective detector coupled to an Agilent 7890 gas chromatograph, equipped with a 30 m × 0.25 mm ×1.0 µm HP-5 MS (5% phenyl-polymethylsiloxane) capillary column. Helium was used as the carrier gas at a linear velocity of 1.0 mL/min. The injector temperature was kept at 220°C and the detector at 280°C. The oven temperature was programmed from 40°C (2 min), increasing at 3°C /min to 150°C (2 min), then increasing at 10 °C /min to 220 °C, and then held for 2 min by optimizing the method used in the previous investigations (*2*, *25*). Mass spectra were recorded in the electron impact (EI) ionization mode at 70 eV. The quadrupole mass detector, ion source and transfer line temperatures were set at 150, 230 and 350°C, respectively. Mass spectra were scanned in the range of *m/z* 30-350 amu at 1 s intervals. Identification of volatile compounds was achieved by comparing the mass spectra of our collected standards and those of the data system library (NIST 05) and linear retention index.

Statistical Analysis

All the cultivars were classified according to two methods: 10 Chinese local (C_l), 14 pure Chinese bred (C_b, cultivars obtained from the cross between two Chinese local cultivars), 20 China × foreign (CF, Chinese bred cultivars containing blood of foreign cultivars), 9 Japanese (J) and 42 American and European cultivars (AE) according to origins; 5 white-flesh flat peaches (WF), 38 white-flesh peaches (WP), 35 yellow-flesh peaches (YP), 3 white-flesh nectarines (WN) and 8 yellow-

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flesh nectarines (YN) according to the color of fruit flesh, the fruit shape and with or without hair on the fruit surface (Table 1).

Data for each cultivar were averages of three replications. A one-way ANOVA analysis was used to determine significant differences of volatiles contents between groups.

Results and Discussion

Identification of Volatiles

Seventy-five volatile compounds were identified and relatively quantified. Some volatiles were only found in a few cultivars in this study (Table 2). Those compounds included 5 C_6 compounds, 8 esters, 13 aldehydes, 8 lactones, 17 terpenoids, 4 ketones, 13 alcohols and 7 other compounds including some carbonyl compounds.

Table 2. Volatiles detected in fruits of all 95 peaches and nectarines

	Compounds[a]	*Codes*	*LRI*[b]
C6 compounds	Hexanal	C1	802
	2-Hexenal	C2	847
	(Z)-3-Hexen-1-ol	C3	851
	(E)-2-Hexen-1-ol	C4	862
	1-Hexanol	C5	870
Lactones	γ-Hexalactone	L1	1051
	γ-Heptalactone	L2	1151
	γ-Octalactone	L3	1255
	γ-Nonalactone	L4	1359
	6-Amyl-α-pyrone	L5	1454
	γ-Decalactone	L6	1464
	δ-Decalactone	L7	1490
	δ-Dodecalactone	L8	1680

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Table 2. (Continued). Volatiles detected in fruits of all 95 peaches and nectarines

	Compounds[a]	*Codes*	*LRI*[b]
Aldehydes	Pentanal	A1	697
	(E)-2-Pentenal	A2	752
	Heptanal	A3	902
	Benzaldehyde	A4	955
	Octanal	A5	1003
	Benzeneacetaldehyde	A6	1039
	(E)-2-Octenal	A7	1056
	Nonanal	A8	1103
	(E)-2-Nonenal	A9	1158
	Decanal	A10	1204
	(E)-2-Decenal	A11	1238
	2,4-Decadienal	A12	1314
	Undecanal	A13	1362
Esters	Butyl acetate	E1	812
	(Z)-3-Hexenyl acetate	E2	1009
	Hexyl acetate	E3	1016
	2-Hexenyl acetate	E4	1018
	Methyl 2-methylpentanoate	E5	1220
	3-Hydroxy-2,4,4-trimethylpentyl 2-methyl-propanoate	E6	1346
	Diisobutyl phthalate	E7	1872
	Dibutyl phthalate	E8	1969
Terpenoids	D-Limonene	T1	1024
	cis-Linalool Oxide	T2	1086
	Linalool	T3	1099
	Oxoisophorone	T4	1140
	(3E)-5-Ethyl-6-methyl-3-hepten-2-one	T5	1145
	Epoxylinalol	T6	1166
	α-Terpineol	T7	1186
	p-Menth-1-en-9-al	T8	1212
	α-Citral	T9	1270

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Table 2. (Continued). Volatiles detected in fruits of all 95 peaches and nectarines

	Compounds[a]	*Codes*	*LRI*[b]
	(E)-Theaspirane	T10	1309
	α -Damascenone	T11	1380
	α, α-Dihydro-á-ionone	T12	1433
	4-(2,6,6-Trimethyl-cyclohex-1-enyl)-butan-2-ol	T13	1441
	Geranyl acetone	T14	1451
	2,6-Ditert-butylquinone	T15	1460
	α -Ionone	T16	1481
	Nerolidol	T17	1747
Alcohols	1-Penten-3-ol	B1	684
	Isoamylol	B2	730
	1-Pentanol	B3	768
	1-Octen-3-ol	B4	980
	2-Ethyl-1-hexanol	B5	1030
	5-Methyl-5-nonanol	B6	1121
	3-Methyl-3-nonanol	B7	1138
	6-Tridecanol	B8	1317
	4-Tridecanol	B9	1339
	10-Dodecyn-1-ol	B10	1446
	6-Pentadecanol	B11	1519
	4-Pentadecanol	B12	1549
	1-Hexadecanol	B13	1594
Ketones	1-Penten-3-one	K1	684
	Cyclohexanone	K2	895
	1-Octen-3-one	K3	978
	6-Methyl-5-hepten-2-one	K4	988

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Table 2. (Continued). Volatiles detected in fruits of all 95 peaches and nectarines

	Compounds[a]	*Codes*	*LRI*[b]
Others	2-Pentyl-furan	O1	991
	Butylated hydroxytoluene	O2	1511
	2,4-Di-tert-butylphenol	O3	1514
	Hexadecane	O4	1600
	Heptadecane	O5	1700
	3,5-Di-tert-butyl-4-hydroxybenzaldehyde	O6	1767
	Nonadecane	O7	1900

[a] Identities confirmed by comparing mass spectra and retention time with those of authentic standards. [b] Linear retention index calculated using a series of *n*- alkane.

Table 3. Average contents of major compounds and the groups of volatiles (μgkg^{-1} FW equivalent of 3-octanol) in different origins of peach and nectarine cultivars

	C_f[a]	C_b	*CF*	*J*	*AE*
C1[c]	128.0±14.4b[b]	145.6±18.3ab	190.0±16.2a	132.2±7.1b	175.9±9.2ab
C2	31.9±9.1	32.2±5.9	38.9±7.1	30.9±5.7	20.5±2.8
C3	20.3±5.2b	38.7±8.7a	38.6±4.9a	29.4±5.0ab	21.3±2.0b
C4	8.3±2.1ab	10.8±1.8ab	12.3±1.8a	9.4±1.2ab	6.6±0.6b
C5	18.9±5.1ab	30.9±5.8ab	33.3±6.0a	27.1±4.4ab	17.9±1.7b
L1	25.0±9.4	23.6±6.3	25.3±4.1	24.2±2.4	22.3±2.0
L5	16.0±4.7b	18.2±4.0b	23.2±6.4ab	42.6±13.4a	21.6±4.4ab
L6	94.0±23.3b	128.0±29.9ab	121.6±27.0ab	201.8±43.2a	96.3±17.6b
L7	37.8±9.0b	52.5±11.8b	50.2±11.2b	87.3±17.9a	37.1±6.8b
A3	7.1±1.4ab	5.8±0.9b	8.2±1.0ab	5.7±1.1b	10.8±1.1a
A4	19.4±2.3	19.1±2.1	26.1±2.1	23.6±6.2	27.0±1.4
A5	9.5±2.3b	6. 8±1.3b	12.1±1.9ab	6.1±1.2b	19.1±1.9a
A7	28.4±3.9bc	26.3±3.4c	37.7±1.9ab	29.5±3.8abc	39.2±2.1a
A8	13.9±1.4c	15.4±0.9bc	20.3±1.2ab	15.8±1.5bc	21.9±1.4a
E1	17.7±1.9	18.5±3.3	22. 9±3.1	14.6±2.5	22.0±1.8
E2	34.5±4.9b	45.8±7.9ab	62.5±6.2a	50.7±7.4ab	61.4±3.7a
E3	5.2±0.9	6.4±1.0	7.7±1.2	6.0±1.1	8.9±1.4

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Table 3. (Continued). Average contents of major compounds and the groups of volatiles (µgkg^{-1} FW equivalent of 3-octanol) in different origins of peach and nectarine cultivars

	C_l^a	C_b	*CF*	*J*	*AE*
E4	4.8±1.4	5.8±0.9	6.4±1.2	5.5±1.0	7.2±1.0
T3	19.5±9.8	11.5±2.9	23.8±6.5	21.3±9.5	35.3±5.7
T7	3.7±1.1b	3.9±0.5b	4.4±0.6b	4.1±0.8b	9.9±1.3a
T8	10.6±4.6ab	7.33±2.5b	11.7±2.8ab	8.3±1.8b	25.0±4.9a
T14	9.4±1.5	10.0±0.8	11.3±1.1	9.7±1.1	9.6±0.7
T16	6.6±1.2bc	11.5±1.4a	10.8±1.7ab	9.2±1.683abc	5.1±0.6c
K4	8.9±1.3	10.4±0.7	11.3±1.1	11.9±1.7	9.3±0.5
O1	7.0±0.9b	6.7±0.7b	11.8±1.7a	7.3±0.9b	11.4±0.5a
O2	29.4±10.1b	51.4±8.5a	43.2±5.2ab	40.2±9.3ab	25.2±3.8b
C6s	205.5±24.4b	258.2±38.9ab	313.2±30.9a	229.0±18.6ab	242.0±13.5ab
C6s % of TV^d	26.80%	28.10%	30.70%	22.90%	26.10%
Lactones	199.6±46.2b	245.0±51.1ab	230.7±47.3ab	381.1±76.9a	195.5±31.0b
Lactones% of TV	26.00%	26.70%	22.70%	38.10%	21.10%
Aldehydes	103.1±12.7b	97.6±10.5b	130.0±9.8ab	96.3±13.3b	151.6±8.6a
Aldehydes% of TV	13.40%	10.60%	12.80%	9.60%	16.30%
Esters	64.5±8.7b	86.3±13.2ab	107.4±10.2a	89.0±10.9ab	103.7±5.7a
Esters% of TV	8.40%	9.40%	10.50%	8.90%	11.20%
Terpenoids	76.8±12.2	70.8±7.2	87.9±8.8	71.4±13.4	116.9±11.5
Terpenoids% of TV	10.00%	7.70%	8.60%	7.10%	12.60%
Alcohols	55.7±7.4b	67.2±5.1ab	67.7±4.3ab	76.9±12.0a	57.2±3.1b
Alcohols% of TV	7.30%	7.30%	6.60%	7.70%	6.20%
Ketones	12.9±1.9	14.3±0.9	15.1±1.5	15.2±2.1	14.3±0.9
Ketones% of TV	1.70%	1.60%	1.50%	1.50%	1.50%
Others	49.6±12.5b	79.0±10.7a	66.4±5.9ab	64.3±10.3ab	46.3±4.3b

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Table 3. (Continued). Average contents of major compounds and the groups of volatiles (μgkg^{-1} FW equivalent of 3-octanol) in different origins of peach and nectarine cultivars

	C_l[a]	*C_b*	*CF*	*J*	*AE*
Others% of TV	6.50%	8.60%	6.50%	6.40%	5.00%
Total	767.7±57.0b	918.4±55.3ab	1018.5±68.7a	1000.3±70.7a	927.5±34.1ab

[a] C_l Chinese local cultivars; C_b pure Chinese original bred cultivars; CF China×foreign cultivars; J Japanese cultivars; AE American and European cultivars. [b] The different small letters indicate significant differences between populations ($P<0.05$). [c] The letter plus the number represents compound corresponding to the code in Table 3. [d] TV = the content of total volatiles.

Table 4. Average contents of major compounds and the groups of volatiles (μgkg^{-1} FW equivalent of 3-octanol) in groups with different fruit characters

	WF[a]	*WP*	*YP*	*WN*	*YN*
C1[c]	279.2±42.7a[b]	147.1±9.1c	148.7±7.6c	211.9±26.4b	205.3±13.5b
C2	94.5±9.6a	31.9±3.6b	17.9±1.9b	24.4±10.6b	21.8±3.4b
C3	85.7±10.6a	29.2±2.6bc	19.0±1.9c	35.8±14.9b	26.8±4.4bc
C4	24.3±4.2a	9.8±0.9b	6.1±0.6b	9.3±1.4b	7.7±1.3b
C5	63.0±16.8a	26.1±2.8b	16.3±1.6b	31.3±9.4b	22.4±3.4b
L1	14.6±5.6	25.4±3.7	24.8±2.5	19.2±5.2	20.0±2.6
L5	30.7±21.9	21.8±3.4	25.9±5.3	16±4.2	17.5±8.2
L6	83.6±39.1	131.7±19.2	122.2±20.1	97.3±8.0	74.2±31.2
L7	46.5±23.7	54.0±8.1	47.1±7.8	34.3±2.5	30.5±10.9
A3	7.7±1.1	6.9±0.8	8.9±0.9	9.4±1.1	13.1±2.4
A4	27.2±2.5ab	21.3±2.0b	24.4±1.3ab	32.7±6.4a	31.0±2.8ab
A5	6.0±0.5b	10.4±1.4b	14.5±1.8ab	15.8±8.3ab	22.2±3.8a
A7	40.1±2.9	29.9±2.4	36.7±1.9	42.5±8.4	40.0±3.0
A8	18.4±1.5	16.9±0.9	19.5±1.3	22.1±6.8	24.1±2.8
E1	30.7±5.5a	17.4±1.8b	19.6±1.4ab	25.7±16.0ab	27.1±4.3ab
E2	95.3±13.4a	50.5±3.8bc	51.8±4.0bc	35.5±6.8c	68.3±6.7b
E3	13.6±2.5a	6.2±0.5ab	6.4±0.7ab	5.7±1.5b	12.8±4.1ab
E4	14.4±3.5a	5.5±0.5b	5.2±0.5b	4.1±0.7b	9.7±2.8ab
T3	64.9±20.8a	18.0±3.9b	31.3±6.1b	10.1±3.2b	27.2±7.4b
T7	5.2±1.0	4.0±0.5	9.0±1.4	5.1±0.7	9.2±2.5

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Table 4. (Continued). Average contents of major compounds and the groups of volatiles (µgkg^{-1} FW equivalent of 3-octanol) in groups with different fruit characters

	WF[a]	*WP*	*YP*	*WN*	*YN*
T8	10.7±2.5	8.9±2.3	21.2±7.0	8.5±1.5	28.9±8.6
T14	12.5±2.5	10.0±0.6	10.3±0.9	12.8±2.7	7.7±1.2
T16	17.6±2.9a	10.7±1.0b	4.7±0.4c	8.6±2.3bc	3.9±0.4c
K4	11.5±1.4.ab	10.8±0.8ab	9.7±0.5ab	13.8±2.8a	7.6±0.7b
O1	10.4±2.0	8.8±1.2	9.9±0.5	13.2±4.1	12.8±1.0
O2	36.2±4.4	47.7±5.5	24.4±3.3	35.5±1.6	24.7±6.1
C6s	546.7±70.5a	243.3±14.5bc	207.9±10.6c	312.6±54.2b	283.4±18.2bc
C6s % of TV[d]	42.1%	26.4%	23.3%	32.9%	29.9%
Lactones	179.1±87.1	247.8±32.5	243.8±36.4	179.9±19.0	163.8±54.7
Lactones% of TV	13.8%	26.9%	27.4%	18.9%	17.3%
Aldehydes	124.4±11.6ab	109.2±8.4b	132.0±7.1ab	156.9±30.2ab	169.4±17.3a
Aldehydes% of TV	9.6%	11.8%	14.8%	16.5%	17.9%
Esters	165.9±21.1a	90.0±6.0b	88.4±6.1b	75.5±20.1b	113.2±11.1b
Esters% of TV	12.8%	9.8%	9.9%	7.9%	12.0%
Terpenoids	142.9±20.0a	80.4±6.3ab	103.3±12.6ab	73.1±5.0b	104.8±17.4ab
Terpenoids% of TV	11.0%	8.7%	11.6%	7.7%	11.1%
Alcohols	66.5±7.5	70.6±4.2	55.6±3.3	73.0±10.1	55.1±3.6
Alcohols% of TV	5.1%	7.7%	6.2%	7.7%	5.8%
Ketones	15.2±3.1	14.7±1.0	13.5±1.0	19.4±3.1	14.4±1.2
Ketones% of TV	1.2%	1.6%	1.5%	2.0%	1.5%
Others	59.2±7.4	72.2±6.5	46.5±4.3	59.6±5.8	43.4±6.1

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Table 4. (Continued). Average contents of major compounds and the groups of volatiles (µgkg^{-1} FW equivalent of 3-octanol) in groups with different fruit characters

	WF[a]	*WP*	*YP*	*WN*	*YN*
Others% of TV	4.6%	7.8%	5.2%	6.3%	4.6%
Total	1299.8±142.3a	922.7±36.8b	891.0±42.1b	950.1±33.8b	947.5±40.5b

[a] WF= white-flesh flat peaches; WP= white-flesh peaches; YP= yellow-flesh peaches; WN= white-flesh nectarines; YN= yellow-flesh nectarines [b] The different small letters indicate significant differences between populations ($P<0.05$). [c] The letter plus the number represents compound corresponding to the code in Table 2. [d] TV = the content of total volatiles.

Total Content of Volatiles

For the groups from different origins, the highest content of the total volatiles was found in the Chinese bred cultivars containing blood of foreign cultivars (CF) and Japanese cultivars (J), which had significantly higher amount than the Chinese local cultivars (C_l) that had the lowest content of volatiles among the five groups (Table 3). Six of the CF, ‘Ruipan 3’ (25), ‘Yangzhou 124 Pantao’ (26), ‘Beijing 5’ (27), ‘Fenglu’ (28), ‘Yihongshuimi’ (34) and ‘Yanfeng’ (38), Five of the Japanese cultivars, ‘Nippon Suimitsu’ (46), ‘Shin Musume’ (48), ‘Ban Ougon’ (50), ‘Kanto 5’ (51), and ‘Okitsu’ (53), had high volatile contents more than 1000 µgkg^{-1} FW (i.e., 1575.4, 1648.2, 1530.6, 1446.4, 1086.3, 1299.5, 1259.5, 1054.5, 1139.5, 1148.3 and 1204.1µgkg^{-1} FW, respectively). The highest and lowest volatile contents in the Chinese local cultivars (C_l) were found in ‘Linbai 4’ (6) and ‘Cuixiangtao’ (4), respectively.

As to the groups with different characters of fruit (Table 4), white-flesh flat peaches (WF) had significantly higher total amounts of volatiles than other groups. Four of the white-flesh flat peaches, including the ‘Ruipan 4’ (11), ‘Ruipan 5’ (12), ‘Ruipan 3’ (25) and ‘Yangzhou 124 Pantao’ (26), had high volatile contents more than 1000 µgkg^{-1} FW (i.e., 1122.4, 1271.6, 1575.4 and 1648.2 µgkg^{-1} FW), respectively.

There were 14 cultivars of AE, including four white-flesh peaches (i.e., ‘Charme’ (54), ‘Corol’ (56), ‘NJ250’ (59) and ‘NJ257’ (60)), six yellow-flesh peaches, (i.e., ‘Babygold 5’ (63), ‘Babygold 6’ (64), ‘Babygold 9’ (65), ‘Dixon’ (68), ‘NJC47’ (78) and ‘Vivian’ (85)), four yellow-flesh nectarines (i.e., ‘Flavortop’ (86), ‘Legrand’ (89), ‘Nectared 6’ (92) and ‘P1’ (93)) and one white-flesh peach with pure Chinese origin, ‘Yutian’ (20), had the volatile contents in more than 1000 µgkg^{-1} FW. In contrast, the cultivars with volatile contents below 600 µgkg^{-1} FW included two white-flesh peaches of C_l , ‘Baiyintao’ (3) and ‘Cuixiangtao’ (4), and three yellow-flesh peaches of AE, ‘Fuzador’ (72), ‘Redtop’ (82) and ‘Riogrand’ (83).

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Composition of Volatiles

The average contents of total volatiles and the main aroma compounds (greater than 5 μgkg^{-1} FW in general) of the fruits are shown based on the origins and characters in Table 3 and Table 4, respectively. The contents of the main volatiles and some special volatiles for all cultivars are given in Table 5.

Table 5. Contents of the major volatiles (μgkg^{-1} FW equivalent of 3-octanol) in the 95 peach and nectarine cultivars

	E2[b]	*A4*	*A7*	*K4*	*T3*	*T10*	*T12*	*T13*	*T16*	*L5*	*L6*	*L7*
1[a]	48.5	21.9	46.0	9.1	91.6	0.6	1.6	0.2	9.9	12.8	30.4	17.1
2	38.4	4.7	4.7	3.2	7.1	2.9	5.9	2.5	7.4	24.0	134.5	73.5
3	27.9	17.3	46.0	4.3	1.3	0.3	0.5	0.3	2.9	0.6	3.2	1.6
4	25.6	24.2	26.1	9.0	1.3	0.5	0.2	-	3.4	0.5	4.9	2.3
5	12.0	16.9	28.0	-[c]	3.6	1.8	5.7	2.2	7.1	27.7	149.4	48.0
6	53.6	17.7	20.0	12.1	3.8	2.0	7.5	2.0	14.6	12.7	137.2	52.7
7	56.3	25.6	34.8	10.9	3.9	0.8	0.6	0.4	3.2	1.7	19.3	4.4
8	23.0	29.1	29.3	13.1	4.1	1.5	6.4	1.5	8.5	49.3	99.2	51.9
9	42.2	24.7	30.9	13.5	18.4	-	-	-	5.9	16.1	204.9	68.8
10	18.1	11.7	18.6	5.4	59.8	-	-	-	2.9	15.0	157.4	57.7
11	94.6	23.7	32.9	10.4	10.2	1.4	2.5	2.0	13.5	2.0	16.1	8.2
12	107.6	35.2	47.7	10.7	24.7	3.7	7.3	3.9	16.2	-	37.8	14.8
13	44.3	11.0	10.8	16.0	2.0	4.9	0.7	-	7.3	7.8	56.8	32.9
14	35.1	26.8	45.3	6.5	37.3	1.6	3.3	2.0	8.7	19.1	92.1	35.0
15	10.5	18.8	29.3	11.2	10.8	0.8	1.9	1.2	10.6	6.5	41.5	34.2
16	41.3	16.6	26.3	7.0	25.5	3.1	5.2	3.2	18.6	12.3	162.7	61.1
17	27.5	12.7	13.7	7.9	3.0	1.7	5.0	1.2	11.0	33.3	210.9	102.5
18	16.5	17.2	26.3	8.7	7.4	3.0	-	3.3	22.1	37.8	203.5	86.2
19	38.4	19.2	25.5	10.4	2.8	0.8	3.6	2.6	15.8	30.3	135.8	70.3
20	30.3	9.4	10.2	14.3	2.1	0.8	7.0	4.5	13.0	47.3	450.5	170.9
21	88.6	7.8	11.7	13.6	1.6	0.8	11.7	6.6	11.3	10.9	115.6	23.0
22	32.9	17.4	24.8	10.4	19.7	-	-	-	3.9	9.8	130.2	38.5
23	42.6	21.5	19.9	10.9	7.2	-	-	-	4.2	17.6	110.6	47.4
24	31.4	31.0	44.2	7.2	7.0	0.9	1.5	1.1	5.3	2.5	28.6	10.4
25	95.4	24.5	36.5	-	121.0	8.0	-	4.2	25.9	96.0	227.0	134.7

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Table 5. (Continued). Contents of the major volatiles (μgkg^{-1} FW equivalent of 3-octanol) in the 95 peach and nectarine cultivars

	E2[b]	*A4*	*A7*	*K4*	*T3*	*T10*	*T12*	*T13*	*T16*	*L5*	*L6*	*L7*
26	130.3	30.5	37.4	15.6	76.9	0.8	9.2	5.1	22.2	11.7	106.7	57.4
27	54.5	25.8	37.5	15.8	26.2	8.3	9.8	6.4	27.5	69.4	345.5	123.9
28	74.0	16.7	16.6	17.8	12.3	4.8	14.0	4.2	13.1	72.2	341.3	174.6
29	65.4	38.4	50.2	8.8	19.4	0.4	0.5	0.1	4.4	1.2	5.9	3.6
30	37.3	26.0	38.4	-	3.7	0.8	1.2	-	9.1	-	19.8	6.8
31	52.2	19.8	30.3	8.2	24.8	-	-	-	6.7	13.1	72.9	25.7
32	64.9	17.0	39.1	6.8	14.6	0.4	1.1	0.2	6.9	4.4	21.8	15.7
33	43.8	4.3	-	2.7	27.2	0.2	15.0	6.2	13.9	10.7	352.2	111.7
34	71.9	32.4	43.1	18.1	3.3	0.8	5.3	3.9	23.3	7.6	62.7	23.5
35	71.3	18.2	33.3	9.9	16.6	-	1.0	-	4.5	-	2.6	1.5
36	62.5	28.7	33.0	7.9	21.4	-	-	-	2.9	1.2	20.8	5.0
37	108.6	30.6	40.1	9.7	10.7	-	0.1	-	4.9	17.4	58.5	30.0
38	77.4	10.9	39.8	-	5.2	-	-	-	9.5	25.8	293.1	95.6
39	32.4	37.0	48.6	17.6	14.0	-	-	-	5.0	18.4	98.6	30.8
40	25.6	41.1	53.1	15.5	3.7	1.7	-	1.0	12.9	21.7	110.4	39.2
41	48.5	20.1	25.9	8.3	12.5	1.8	9.2	2.6	7.9	7.9	82.8	33.0
42	26.2	29.5	33.7	12.8	2.0	-	-	-	5.9	22.0	132.5	47.0
43	30.0	39.8	42.3	10.3	5.5	-	-	-	5.3	15.0	66.5	34.4
44	78.0	30.7	37.9	6.1	56.1	-	-	-	3.7	2.4	10.3	9.4
45	38.1	21.8	31.6	9.0	18.9	0.5	3.7	1.0	5.7	8.2	49.1	43.3
46	65.4	25.5	45.9	20.3	82.0	2.7	8.4	5.2	15.1	36.0	170.9	85.8
47	23.5	21.1	26.1	15.7	0.8	1.5	-	2.0	7.4	11.8	71.9	27.2
48	87.5	69.8	28.7	17.9	3.6	0.8	5.2	2.7	9.8	14.6	101.6	36.0
49	77.8	8.3	-	9.5	9.6	-	14.4	-	19.8	9.9	213.2	68.6
50	46.9	16.0	27.6	9.6	15.9	-	-	-	7.1	60.8	188.0	159.3
51	26.6	12.1	22.2	7.8	36.5	-	-	-	5.4	89.6	346.5	142.1
52	52.4	29.0	42.6	12.7	3.2	-	0.3	-	5.7	30.8	225.4	61.3
53	38.1	8.4	11.5	4.3	-	-	-	-	6.3	122.0	449.2	162.0
54	55.3	9.9	9.7	3.4	81.4	3.1	-	2.9	13.7	55.7	339.4	192.9
55	47.3	11.7	15.7	14.2	91.3	7.0	10.6	3.8	18.0	9.5	297.3	72.0
56	43.3	37.7	30.9	12.0	5.0	1.1	5.0	2.0	6.0	9.6	64.6	24.8

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Table 5. (Continued). Contents of the major volatiles (μgkg^{-1} FW equivalent of 3-octanol) in the 95 peach and nectarine cultivars

	E2[b]	*A4*	*A7*	*K4*	*T3*	*T10*	*T12*	*T13*	*T16*	*L5*	*L6*	*L7*
57	33.9	15.8	23.9	6.0	37.3	13.3	16.5	7.8	21.6	64.3	240.2	89.1
58	86.9	25.4	43.6	10.5	5.1	1.4	2.2	1.4	4.6	12.6	68.0	18.1
59	92.2	35.7	73.1	15.3	13.7	1.0	2.6	0.8	4.4	7.0	21.5	7.7
60	67.7	32.5	55.7	9.6	68.2	0.8	2.2	0.9	4.8	24.1	121.9	46.4
61	95.4	19.1	16.8	-	2.7	-	1.0	-	2.4	-	1.6	1.2
62	61.0	33.5	58.2	9.4	11.5	-	-	-	2.7	2.1	10.3	3.6
63	63.8	19.5	29.4	13.0	37.7	-	-	-	11.6	105.4	360.9	129.5
64	47.6	8.4	15.6	7.1	89.9	-	-	-	8.4	140.3	503.0	185.6
65	59.3	29.6	37.9	9.7	85.9	-	-	-	3.3	27.7	86.0	31.3
66	45.1	21.3	28.5	-	62.5	-	-	-	2.0	6.3	26.9	13.2
67	17.3	27.6	41.8	8.3	2.1	-	-	-	2.6	8.5	37.4	15.4
68	45.0	29.2	48.1	16.5	12.6	-	-	-	9.3	37.7	235.9	62.6
69	47.3	36.8	67.6	9.7	20.0	-	-	-	3.6	17.0	58.2	29.3
70	48.5	21.0	25.9	5.4	18.9	-	-	-	2.3	9.9	47.7	16.3
71	66.9	26.3	32.4	11.3	2.4	-	-	-	2.6	3.6	15.7	5.9
72	27.9	27.5	34.7	8.4	3.0	-	-	-	3.3	5.8	47.3	13.3
73	44.7	23.9	35.9	13.1	40.0	-	-	-	4.8	48.8	177.5	66.8
74	66.7	18.5	37.9	11.2	33.7	-	-	-	5.2	40.7	101.3	58.2
75	40.1	25.9	53.8	10.8	6.1	-	-	-	2.4	5.7	21.0	7.6
76	112.7	35.3	48.7	8.3	13.8	-	-	-	4.0	7.3	42.6	15.1
77	72.7	34.0	50.7	13.0	7.0	-	-	-	6.7	12.8	47.4	32.9
78	48.2	24.0	36.3	11.5	29.2	-	-	-	6.0	21.6	222.6	73.0
79	45.5	20.3	22.8	7.5	40.0	-	-	-	5.9	58.3	190.7	64.1
80	40.0	19.9	40.0	7.1	89.9	-	-	-	3.5	23.5	124.6	42.8
81	71.5	27.7	34.8	9.1	31.4	-	-	-	3.8	10.3	40.3	15.1
82	38.2	19.2	39.6	6.3	16.7	-	-	-	2.2	11.9	34.6	17.1
83	19.3	22.0	33.7	6.0	49.8	-	-	-	2.3	6.7	40.3	17.6
84	109.8	40.4	48.1	13.0	3.9	-	-	-	4.1	7.0	28.8	9.7
85	32.9	28.0	38.4	-	182.2	-	-	-	2.9	2.7	11.2	6.3
86	101.0	35.8	50.7	7.0	78.4	-	-	-	3.1	3.6	14.7	8.3
87	81.2	27.7	42.4	6.3	5.2	-	-	-	2.1	1.4	4.6	3.2

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Table 5. (Continued). Contents of the major volatiles (μgkg^{-1} FW equivalent of 3-octanol) in the 95 peach and nectarine cultivars

	E2[b]	*A4*	*A7*	*K4*	*T3*	*T10*	*T12*	*T13*	*T16*	*L5*	*L6*	*L7*
88	57.1	26.8	36.4	5.5	16.8	-	-	-	3.6	3.2	11.4	4.4
89	86.9	34.2	50.8	5.4	55.8	-	-	-	2.8	11.4	33.6	19.8
90	61.5	21.2	25.7	11.9	2.8	-	-	-	4.4	9.5	74.4	29.1
91	89.7	36.4	54.6	6.0	8.8	-	-	-	3.1	0.5	2.6	1.0
92	69.3	19.6	37.4	-	3.8	-	-	-	5.1	19.1	136.2	46.3
93	60.9	46.4	45.1	8.2	46.0	-	-	-	3.4	15.6	45.7	22.5
94	69.0	29.8	51.0	7.9	52.8	-	-	-	2.3	9.0	8.7	23.2
95	107.8	47.7	40.7	7.1	19.2	-	-	-	3.7	10.4	48.7	17.2

[a] Numbers 1-95 represent the cultivars responding to the accession number in Table 1. [b] The letter plus the number represents compound corresponding to the code in Table 2. [c] Not detected in sample.

C_6 Compounds

Five C_6 compounds including hexanal (C1), 2-hexenal (C2), (*Z*)-3-hexen-1-ol (C3), (*E*)-2-hexen-1-ol (C4), and 1-hexanol (C5) were found (Table 2). Among them, hexanal (C1) and 2-hexenal (C2) were the major C_6 compounds (Table 3, 4).

C_6 compounds in all cultivars from the five origins contributed 22.9-30.7% of the total volatile content. The sum of C_6 compounds was significantly higher in CF than in C_l (Table 3). As to the groups with different fruit characters, C_6 compounds contributed 23.3-42.1% of the total volatile content. The level of C_6 compounds in white-flesh flat peaches (WF) was significantly higher than that in other groups. Yellow-flesh peaches (YP) had the lowest contents of the C_6 compounds, which were significantly lower than those in the white-flesh nectarines (WN) (Table 4).

Being described as having a 'grassy' flavor (*7*), C_6 compounds are the major compounds in immature peaches and nectarines; however, their levels decrease along with the maturity of the fruits (*12*, *13*, *16*). High content of C_6 compounds was found in our previous work (*11*). Relatively less C_6 compounds were found in this work probably because of the addition of $CaCl_2$ that prevented the enzymatic breakdown of the unsaturated fatty acids which are known as precursors of the C_6 compounds.

Lactones

Eight lactones were identified, and ranked the second abundant group of volatiles accounting for 21.1-38.1% of the total volatiles (Table 3). In comparison with our previous work, 6-amyl-α-pyrone (L5) and δ-dodecalactone (L8) were

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found this time, whist δ-octalactone and 7-decen-5-olide could not be detected (Table 2) (*11*). In all groups, 6-amyl-α-pyrone (L5), γ-decalactone (L6) and δ-decalactone (L7) were the major components accounting for 74.1-87.0% of the total lactonic compounds, and γ-decalactone (L6) was the dominant (Table 3, 5).

The sum of lactones was significantly higher in the Japanese cultivars than in C_l and AE (Table 3). However, there was no significant difference among groups with different fruit characters (Table 4). As the most abundant lactone, γ-decalactone (L6) in the Japanese cultivars was significantly in higher content than in C_l and AE (Table 3).

Contents of 6-amyl-α-pyrone (L5), γ-decalactone (L6) and δ-decalactone (L7) were high in eight cultivars with different origins, including ‘Ruipan 3’ (25), ‘Beijing 5’ (27), ‘Fenglu’ (28), ‘Kanto 5’ (51), ‘Okitsu’ (53), ‘Charme’ (54), ‘Babygold 5’ (63) and ‘Babygold 6’ (64), and one pure Chinese original cultivar, ‘Yutian’ (20), with more than 50, 200, 100 μgkg-1 FW, respectively (Table 5).

6-Amyl-α-pyrone (L5) was found in peaches and nectarines in a few previous studies by means of liquid-liquid extraction and capillary separation (*4*, *13*). It is the first time that L5 was detected by HS-SPME-GC-MS installed with the HP-5 MS capillary column. There was no 6-amyl-α-pyrone (L5) in the ‘Ruipan 5’ (12), ‘Jingyu’ (30), ‘Zaoyu’ (35) and ‘P6’ (61) (Table 5).

Lactones, particularly γ-decalactone and δ-decalactone, have been reported as “character impact” compounds of the peach and nectarine aroma. They act in association with other volatiles, such as C_6 aldehydes, C_6 alcohols and terpenoids, to produce the specific flavors of peach and nectarine. Particularly, lactones contribute the “peachy” background whilst others contribute fruity and floral notes (*14*, *16*). Although it was reported that nectarines contained significantly higher amounts of δ-decalactone than peaches (*5*, *13*), flat peaches was found to have significantly higher content of δ-decalactone than yellow-flesh nectarines, and white- and yellow-flesh peaches in our previous study. In this work, no significant difference of the content of δ-decalactone was found between flat peaches, peaches and nectarines (Table 4). Such quantitative discrepancy between different studies were attributed to the small number of studied germplasms in our previous studies.

Aldehydes

Among the detected 13 aldehydes, heptanal (A3), benzaldehyde (A4), octanal (A5), (E)-2-octenal (A7) and nonanal (A8) were major aldehydes (Table 3). In this work, (E)-2-octenal (A7) was found to be the most abundant aldehyde, while benzaldehyde was the dominant aldehyde in our previous work and other studies (*11*, *14*, *16*).

The sum of the aldehydes in AE was significantly higher than in C_l, C_b and J (Table 3). The aldehyde level in YN was significantly higher than in WP (Table 4). Ten AE cultivars including one white-flesh peach (i.e.,‘NJ250’ (59)), three yellow-flesh peaches (i.e., ‘Androreda’ (62), ‘Loring’ (76) and ‘Shasta’ (84)), and six yellow-flesh nectarines (i.e., ‘Flavortop’ (86), ‘Legrand’ (89), ‘Nectared 4’

(91), ‘P1’ (93), ‘Tasiva’ (94) and ‘Vega’ (95)) were the cultivars with aldehyde content of more than 200 μgkg^{-1} FW (Table 5).

Esters

Eight esters were found in peaches and nectarines in this study (Table 2). Butyl acetate (E1), (*Z*)-3-hexenyl acetate (E2), hexyl acetate (E3) and 2-hexenyl acetate (E4) were the major esters accounting for 87.5-96.6% of total amount of esters (Table 3).

Esters are considered to be main contributors to fruity and floral notes. High content in esters should give a pleasant flavor in peaches and nectarines (*22*). As the major components, esters contributed to 8.4-11.2% of the total volatiles (Table 3). Among the five groups, the sum of the esters in CF and AE were significantly higher than in C_l. This result is consistent to our previous report that American and European cultivars had higher ester contents (*11*). Regarding the groups with different fruit characters, esters contributed 7.9-12.8% to the total volatile content. Esters content of WF was significantly higher than that of other groups because of the high contents of the most abundant ester, ‘(*Z*)-3-hexenyl acetate’ (E2), in four WF: ‘Ruipan 4’ (11), ‘Ruipan 5’ (12), ‘Ruipan 3’ (25) and ‘Yangzhou 124 Pantao’ (26, Table 4, 5). The contents of ‘(*Z*)-3-hexenyl acetate’ (E2) in one YP of the CF, ‘Qiukui’ (37), and six AE cultivars, ‘NJ250’ (59), ‘P6’ (61), ‘Loring’ (76), ‘Shasta’ (84), ‘Flavortop’ (86), and ‘Vega’ (95) were also above 90 μgkg^{-1} FW (Table 5).

Terpenoids

Among the seventeen terpenoids that were identified in this investigation (Table 2), linalool (T3), α-terpineol (T7) and p-menth-1-en-9-al (T8) were the most abundant compounds (Table 3).

The total content of terpenoids accounted for 7.1-12.6% of the total amount of volatiles (Table 3). The sum of terpenoids in AE was higher than in other four groups but there was no significant difference among them (Table 3).

Terpenoids contributed 7.7-11.6% to total volatile content. Terpenoids in WF was significantly higher than in WN because of the high contents of the most abundant terpenoid, linalool (T3), in three WF, ‘Baimangpantao’ (1), ‘Ruipan 3’ (25) and ‘Yangzhou 124 Pantao’ (26) (Table 4, 5). One yellow-flesh peach of AE, Vivian (85), had very high contents of linalool (T3), α-terpineol (T7) and p-menth-1-en-9-al (T8). Other cultivars with high content of linalool (T3) and terpenoids were (Table 5): three white-flesh peaches, ‘Nippon Suimitsu’ (46), ‘Corol’ (56) and ‘NJ257’ (60), three yellow-flesh peaches, ‘Babygold 6’ (64), ‘Babygold 9’ (65) and ‘NJC237’ (80), and four yellow-flesh nectarines, ‘Flavortop’ (86), ‘Legrand’ (89), ‘P1’ (93) and ‘Tasiva’ (94).

Three terpenoid compounds, (E)-theaspirane (T10), α,α-dihydro-α-ionone (T12) and 4-(2,6,6-trimethyl-cyclohex-1-enyl)-butan-2-ol (T13), were found only in some cultivars of C_l, C_b, CF, J and white-flesh peaches of AE. They were not

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detected in the yellow-flesh peaches and yellow-flesh nectarines of AE. It is in agreement with the result in our previous work that no α,α-dihydro-α-ionone was detected in yellow-flesh peaches and yellow-flesh nectarines of American and European cultivars (*11*).

Terpenoids contribute fruity characteristics to peach (*13*). For example, linalool, which increases significantly during the fruit maturation, is considered one of the major aroma contributors to the ripen peaches and nectarines (*3*, *9*, *16*). Yet, as it was reported in our previous research, not all the nectarines had high linalool level and there was no significant difference between white- and yellow-fleshed peaches.

Ketones, Alcohols, and Other Compounds

In this work, 4 ketones, 13 alcohols and othe 7 miscellaneous carbonyl compounds were found. The sum of ketones, alcohols and other carbonyl compounds accounted for 1.5-1.7%, 6.2-7.7% and 5.0-8.6% of the total volatiles, respectively. (Table 3). They are not discussed here in detail.

Conclusions

Composition and content of volatile chemicals in different peaches and nectarines depended largely upon genetic background. China × foreign and Japanese cultivars had significantly higher contents of the total volatiles and esters than the Chinese local cultivars. White-flesh flat peaches had the highest total amount of volatiles and esters, which were significantly higher than other groups with different fruit characters. Terpenoids in white-flesh flat peaches was significantly higher than in white-flesh nectarines. Ten cultivars with high contents of lactones, terpenoids and total volatiles, such as ‘Yutian’, ‘Ruipan 3’, ‘Yangzhou 124pantao’, ‘Beijing 5’, ‘Fenglu’, ‘Kanto 5’, ‘Okitsu’, ‘Charme’, ‘Babygold 5’ and ‘Babygold 6’, could be chosen as the desirable cultivars for breeding.

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References

1. Aubert, C.; Günata, Z.; Ambid, C.; Baumes, R. Changes in physicochemical characteristics and volatile constituents of yellow and white-fleshed

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Publication Date (Web): March 18, 2010 | doi: 10.1021/bk-2010-1035.ch007

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Publication Date (Web): March 18, 2010 | doi: 10.1021/bk-2010-1035.ch007

nectarines during maturation and artificial ripening. *J. Agric. Food Chem.* **2003**, *51*, 3083–3091.

2. Aubert, C.; Milhet, C. Distribution of the volatile compounds in the different parts of a white-fleshed peach (*Prunus persica* L. Batsch). *Food Chem.* **2007**, *102*, 375–384.
3. Chapman, G. W., Jr.; Horvat, R. J.; Forbus, W. R., Jr. Physical and chemical changes during the maturation of peaches (cv. Majestic). *J. Agric. Food Chem.* **1991**, *39*, 867–870.
4. Derail, C.; Hofmann, T.; Schieberle, P. Difference in key odorants of handmade juice of yellow-flesh peaches (*Prunus persica* L.) induced by the workup procedure. *J. Agric. Food Chem.* **1999**, *47*, 4742–4745.
5. Engel, K. H.; Ramming, D. W.; Flath, R. A.; Teranashi, R. Investigation of volatile constituents in nectarines. 2. Changes in aroma composition during nectarine maturation. *J. Agric. Food Chem.* **1988**, *36*, 1003–1006.
6. Jia, H. J.; Okamoto, G.; Hirano, K. Studies on the sensory evaluation of juice constituents of peach fruit. *J. Fruit Sci.* **2004**, *21*, 5–10.
7. Kakiuchi, N.; Ohmiya, A. Changes in the composition and content of volatile constituents in peach fruits in relation to maturity at harvest and artificial ripening. *J. Jpn. Soc. Hortic. Sci.* **1991**, *60*, 209–216.
8. Lavilla, T.; Recasens, I.; Lopez, M. L. Production of volatile aromatic compounds in Big Top nectarines and Royal Glory peaches during maturity. *Acta Hortic.* **2001**, *553*, 233–234.
9. Robertson, J. A.; Meredith, F. I.; Horvat, R. J.; Senter, S. D. Effect of cold storage and maturity on the physical and chemical characteristics and volatile constituents of peaches (cv. Cresthaven). *J. Agric. Food Chem.* **1990**, *38*, 620–624.
10. Takeoka, G. R.; Flath, R. A.; Guntert, M.; Jennings, W. Nectarine volatiles: Vacuum steam distillation versus headspace sampling. *J. Agric. Food Chem.* **1988**, *36*, 553–560.
11. Wang, Y. J.; Yang, C. X.; Li, S. H.; Yang, L.; Wang, Y. N.; Zhao, J. B.; Jiang, Q. Volatile Characteristics of 50 Peaches and Nectarines Evaluated by HP-SPME with GC-MS. *Food Chem.* **2009**, *116*, 356–364.
12. Do, J. Y.; Salunkhe, D. K.; Olson, L. E. Isolation, identification and comparison of the volatiles of peach fruit as related to harvest maturity and artificial ripening. *J. Food Sci.* **1969**, *34*, 618–621.
13. Engel, K. H.; Flath, R. A.; Buttery, R. G.; Mon, T. R.; Ramming, D. W.; Teranashi, R. Investigation of volatile constituents in nectarines. 1. Analytical and sensory characterization of aroma components in some nectarine cultivars. *J. Agric. Food Chem.* **1988**, *36*, 549–553.
14. Horvat, R. J.; Chapman, G. W.; Robertson, J. A.; Meredith, F. I.; Scorza, R.; Callahan, A. M.; Morgens, P. Comparison of the volatile compounds from several commercial peach cultivars. *J. Agric. Food Chem.* **1990**, *38*, 234–237.
15. Visai, C.; Vanoli, M. Volatile compound production during growth and ripening of peaches and nectarines. *Sci. Hort.* **1997**, *70*, 15–24.

16. Horvat, R. J.; Chapman, G. W. Comparison of volatile compounds from peach fruit and leaves (cv. Monroe) during maturation. *J. Agric. Food Chem.* **1990**, *38*, 1442–1444.
17. Watada, A. E.; Anderson, R. E.; Aulenbach, B. B. Sensory, compositional, and volatile attributes of controlled atmosphere stored peaches. *J. Am. Soc. Hortic. Sci.* **1979**, *104*, 626–629.
18. Jia, H. J.; Hirano, K.; Okamoto, G. Effects of fertilizer levels on tree growth and fruit quality of 'Hakuho' peaches (*Prunus persica*). *J. Jpn. Soc. Hortic. Sci.* **1999**, *68*, 487–493.
19. Jia, H. J.; Araki, A.; Okamoto, G. Influence of fruit bagging on aroma volatiles and skin coloration of 'Hakuho' peach (*Prunus persica* Batsch). *Postharvest Biol. Tech.* **2005**, *35*, 61–68.
20. Génard, M.; Bruchou, C. Multivariate analysis of within-tree factors accounting for the variation of peach fruit quality. *Sci. Hort.* **1992**, *52*, 37–51.
21. Meredith, F. I.; Robertson, J. A.; Horvat, R. J. Changes in physical and chemical parameters associated with quality and postharvest ripening of Harvester peaches. *J. Am. Soc. Hortic. Sci.* **1989**, *37*, 1210–1214.
22. Sumitani, H.; Suekane, S.; Nakatani, A.; Tatsuka, K. Changes in composition of volatile compounds in high pressure treated peach. *J. Agric. Food Chem.* **1994**, *42*, 785–790.
23. Robertson, J. A.; Horvat, R. J.; Lyon, B. G.; Meredith, F. I.; Senter, S. D.; Okie, W. R. Comparison of quality characteristics of selected yellow- and white-freshed peach cultivars. *J. Food Sci.* **1990**, *55*, 1308–1311.
24. Guillot, S.; Peytavi, L.; Bureau, S.; Boulanger, R.; Lepoutre, J. P.; Crouzet, J.; Schorr-Galindo, S. Aroma characterization of various apricot varieties using headspace–solid phase microextraction combined with gas chromatography–mass spectrometry and gas chromatography–olfactometry. *Food Chem.* **2006**, *96*, 147–155.
25. Riu-Aumatell, M.; Castellari, M.; López-Tamames, E.; Galassi, S.; Buxaderas, S. Characterisation of volatile compounds of fruit juices and nectars by HS/SPME and GC/MS. *Food Chem.* **2004**, *87*, 627–637.
26. Beaulieu, J. C.; Grimm, C. C. Identification of volatile compounds in cantaloupe at various developmental stages using solid phase microextraction. *J. Agric. Food Chem.* **2001**, *49*, 1345–1352.
27. Servili, M.; Selvaggini, R.; Taticchi, A.; Begliomini, A. L.; Montedoro, G. Relationships between the volatile compounds evaluated by solid phase microextraction and the thermal treatment of tomato juice: Optimization of the blanching parameters. *Food Chem.* **2000**, *71*, 407–415.
28. Ibañéz, E.; López-Sebastián, S.; Ramos, E.; Tabera, J.; Reglero, G. Analysis of volatile fruit components by headspace solid-phase microextraction. *Food Chem.* **1998**, *63*, 281–286.
29. Jia, M.; Zhang, Q. H.; Min, D. B. Optimization of solidphase microextraction analysis for headspace flavor compounds of orange juice. *J. Agric. Food Chem.* **1998**, *46*, 2744–2747.

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Publication Date (Web): March 18, 2010 | doi: 10.1021/bk-2010-1035.ch007

30. Song, J.; Gardner, B. D.; Holland, J. F.; Beaudry, R. M. Rapid analysis of volatile flavor compounds in apple fruit using SPME and GC/time-of-flight mass spectrometry. *J. Agric. Food Chem.* **1997**, *45*, 1801–1807.
31. Zhang, Z.; Pawliszyn, J. Headspace solid phase microextraction. *Anal. Chem.* **1993**, *65*, 1843–1852.
32. Gonçalves, C.; Alpendurada, A. F. Comparison of three different poly (dimethylsiloxane)-divinylbencene fibres for the analysis of pesticide multiresidues in water simples: Structure and efficiency. *J. Chromatogr., A* **2002**, *963*, 19–26.
33. Li, S. H.; Zhang, X. P.; Meng, Z.Q.; Wang, X. Responses of peach trees to modified pruning. I. Vegetative growth. *N. Z. J. Crop Hortic. Sci.* **1994**, *22*, 401–409.
34. Page, B. D; Lacroix, G. Application of solid phase microextraction to the headspace gas chromatography analysis of halogenated volatiles in selected foods. *J. Chromatogr., A.* **1993**, *648*, 199–211.
35. Buttery, R. G.; Terahnishi, R.; Ling, L. C.; Flath, R. A.; Stern, D. J. Fresh tomato volatiles: A quantitative study. *J. Agric. Food Chem.* **1987**, *35*, 540–544.
36. Ceva-Antunes, P. M. N.; Bizzo, H. R.; Silva, A. S.; Carvalho, C. P. S.; Antunes, O. A. C. Analysis of volatile composition of siriguela (*Spondias purpurea* L.) by solid phase microextraction (SPME). *LWT-Food Sci. Technol.* **2006**, *39*, 436–442.

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Chapter 8

Health Benefits of Berries for Potential Management of Hyperglycemia and Hypertension

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Many studies have suggested that major chronic diseases, including type 2 diabetes, cardiovascular diseases and certain types of cancer are influenced by dietary and lifestyle factors. It is projected that the total worldwide mortality attributed to inadequate intake of fruits and vegetables is around 2.6 million deaths per year. The prevalence of diagnosed diabetes has increased dramatically over the past 40 years globally and is projected to reach 400 million by 2030. In the USA, diabetes is the fifth leading cause of death with 90% of the cases being adult onset type 2 diabetes. Thus, there is a need to investigate the biochemical and metabolic relevance of readily available fruits and vegetables commonly consumed by the population which could help in the overall management of type 2 diabetes, along with life style changes and medications when relevant. In this context, berry fruits and berry-derived products are commonly consumed in the diet and are considered one of the best dietary sources of phenolic compounds which are associated with several potential health benefits related to management of type 2 diabetes and its complications.

Biosynthesis and Function of Phenolic Compounds in Fruits

Phenolic compounds are secondary metabolites that are derivatives of the pentose phosphate, shikimate and phenylpropanoid pathways in plants

In Flavor and Health Benefits of Small Fruits; Qian, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

(*1*) (Figure 1), comprising an aromatic ring, bearing one or more hydroxyl substituents, and ranging from simple phenolic molecules to highly polymerized compounds (*2*) (Figure 2). The first step in the synthesis of phenolic compounds is the commitment of glucose to the pentose phosphate pathway (PPP), converting glucose-6-phosphate irreversibly to ribulose-5-phosphate. This first committed step in the conversion to ribulose-5-phosphate is carried out by glucose-6-phosphate dehydrogenase (G6PDH). The conversion to ribulose-5-phosphate also produces reducing equivalents (NADPH) for cellular anabolic reactions. PPP also generates erythrose-4-phosphate which along with phosphoenolpyruvate, from glycolysis, is channeled to the shikimate pathway to produce phenylalanine, which is directed through the phenylpropanoid pathway to produce phenolic compounds (*3*–*7*) (Figure 1).

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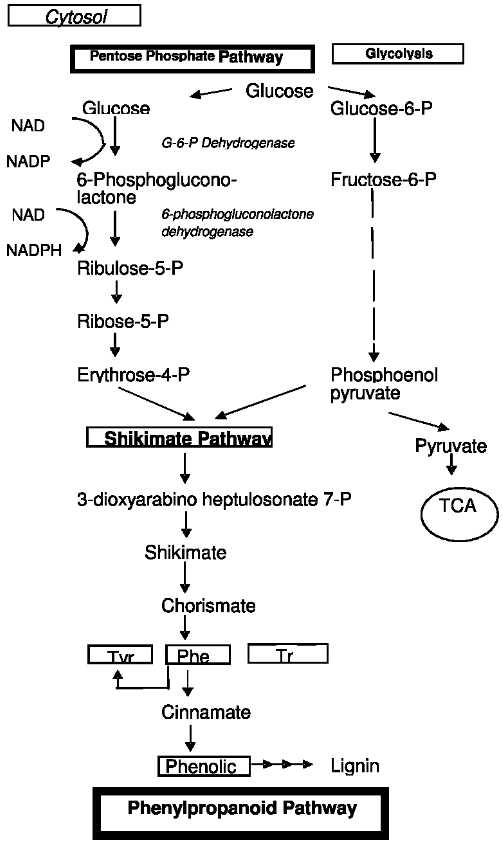
Cytosol
Pentose Phosphate Pathway
Glycolysis
Glucose
Glucose
Glucose-6-P
NAD
NADP
G-6-P Dehydrogenase
6-Phosphoglucono-lactone
Fructose-6-P
NAD
NADPH
6-phosphogluconolactone dehydrogenase
Ribulose-5-P
Ribose-5-P
Erythrose-4-P
Phosphoenol pyruvate
Shikimate Pathway
Pyruvate
3-dioxyarabino heptulosonate 7-P
TCA
Shikimate
Chorismate
Tyr
Phe
Tr
Cinnamate
Phenolic
Lignin
Phenylpropanoid Pathway

Figure 1. Biosynthesis of phenolic compounds. (Adapted from Shetty et al., 2008). (24).

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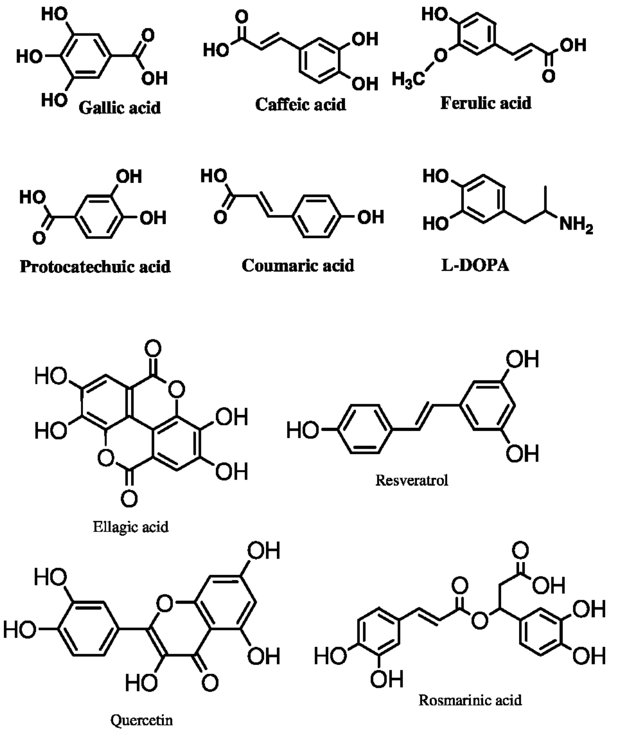

Figure 2. Common phenolic compounds in plants. (Adapted from Shetty et al., 2008). (24).

More than 80,000 different plant phenolic secondary metabolites have been described and these compounds are generally associated with defense responses in the plant. However, other roles are also attributed to phenolic metabolites including attractants to promote pollination, coloring to camouflage or attraction, antibacterial or antifungal and defense against herbivores (*1*, *8–10*). Parr and Bolwell (*11*) reported that the phenolic compounds can act as structural polymers (lignin), UV screens (flavonoids), antioxidants, attractants (flavonoids and carotenoids), defense response (tannins, phytoalexins) and signal compounds (salicylic acid, flavonoids). In addition, these compounds have important functions related to the quality characteristics of fruits and vegetables such as appearance, flavour and health-promoting properties (*12*).

It is known that the biosynthesis of phenolic compounds is stimulated in plants by exposure to high levels of solar radiation (*13–15*). Sullivan *et al.* (*16*) reported that plants growing at high altitudes in tropical mountains, e.g. naturally exposed to high levels of UV radiation, accumulate phenolics in the leaves. The content of phenolic compounds in fruits varies according to the degree of maturity, genetic differences (cultivar), environmental conditions, post harvest storage conditions and processing (*17–19*).

Phenolic compounds, including stress-linked phytochemicals have been associated with the beneficial effects derived from the consumption of fruits and vegetables (*20*), especially due to their antioxidant activity (*21*). Balasundram *et al.* (*22*) reviewed the antioxidant activity, occurrence and potential uses of phenolic compounds in plants and agri-industrial by-products. According to these authors, fruits, vegetables and beverages are the major sources of phenolic compounds in the human diet. Commonly consumed fruits such as apples, cranberries, grapes, raspberries and strawberries and their derived-products like juices and jams are well known as rich sources of phenolic compounds (*4*, *23*).

Health Benefits of Phenolic Compounds

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Reactive oxygen (ROS) and nitrogen (RNS) species are highly reactive oxidized molecules that are constantly produced under normal cellular conditions such as during the activity of mithocondrial respiratory chain and inflammation which could lead to damage in other biological molecules like DNA and proteins (*25–27*) (Figure 3). The antioxidant enzymes-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) play an important role in scavenging these oxidants and preventing cellular injury. Many studies have reported the benefits of phenolic compounds as antioxidant, anti-inflammatory, anti-aging and antiproliferative agents besides the modulation of the above and related antioxidant enzymes to counter oxidants (*28–32*). In addition, phenolic compounds present in fruits, vegetables, berries, beverages and herbal medicines could promote health benefits by reducing the risk of the metabolic syndrome and related complications of type 2 diabetes (*33*). These effects are attributed in general to the potential ability of the phenolic compounds to reduce, counteract or also repair damage resulting from oxidative stress and inflammation associated with these diseases conditions.

Many studies have associated the increase in the consumption of fruits and vegetables containing high levels of antioxidant compounds with the reduction on the risk of certain chronic diseases such as cardiovascular diseases and diabetes (*34–38*). Berries are considered good sources of phenolic compounds such as flavonoids and phenolic acids. Shukitt-Hale *et al.* (*31*) suggested that nutritional interventions containing phenolic compounds from berry fruits could be considered a valuable tool to prevent the development of age-related neurodegenerative diseases by reducing oxidative stress and inflammation.

According to Seeram (*39*) there is a wide range of observed biological properties related to the phenolic compounds present in berry fruits and they may be correlated to the type of individual phenolics rather than the total phenolic content. Therefore, there is a need of more in-depth studies into the bioavailability and metabolism of these compounds since the *in vitro* antioxidant activity does not reflect *in vivo* biological activity. Also, there is a need to take into consideration the individual to individual variability for metabolism and absorption of phenolic compounds. Further it is known that many phenolic compounds are metabolized by the microbiota in the colon, leading to differences in the rate of metabolism and products formed (*40*).

Type 2 Diabetes and Related Hypertension Complications

Several factors such as population growth, aging, urbanization, use of tobacco, absence of physical activity and consumption of unhealthy refined high calorie foods are implicated in the increasing numbers of individuals affected by type 2 diabetes and complications leading to heart disease and stroke (*41*, *42*). According to Winer and Sowers (*43*) the prevalence of diagnosed diabetes has increased over the past 40 years worldwide although in the past decades there was a significant improvement in health care globally. However, the abundance of overly rich and refined macronutrients, the availability of "fast food" and the marketing around the food products have contributed to the increase on the prevalence of overweight US children and adults and this trend is being followed globally. Around 400 million people are projected to have type 2 diabetes globally by 2030 (*41*). According to King *et al.* (*44*) by 2025, India, China and United States will be the countries with the largest number of people with diabetes.

The common forms of *diabetes mellitus* are type 1 and type 2 diabetes, the latter being the more prevalent form and generally appears later in life. In type 2 diabetes, after some period of time the pancreatic β-cells are not capable of augmenting the secretion of insulin and thereby the overall insulin secretion declines, resulting in insulin deficiency (*45*). It is known that oxidative stress and inflammation can lead to the development of obesity-related insulin resistance (*33*) (Figure 4). In addition, hyperglycemia and dyslipidemia can induce inflammatory responses and generation of free radicals which leads to type diabetes and cardiovascular complications (*46*, *47*). Hyperglycemia has been reported to cause increased production of oxygen free radicals through glucose auto-oxidation and nonenzymatic glycation (Maillard reaction) processes, and, consequently, may damage cellular and tissue components (*43*, *48*).

Commercial drugs such as α-glucosidase inhibitors (Acarbose and Miglitol) available in the market are normally prescribed for the treatment of obesity and diabetes. The therapeutic approach available for managing type 2 diabetes is by retarding the absorption of glucose through the reduction of starch hydrolysis by mildly inhibiting pancreatic αamylase and strongly inhibiting intestinal glucose absorption by αglucosidase enzymes. Although pharmacological therapy for metabolic syndrome is available, the role of such therapy in the absence of lifestyle changes should be considered (*49*). Thus, Pan *et al.* (*50*) studied the effect of including changes in diet and exercise and the reduced risk for development of diabetes. These authors observed a reduction of 31% when the diet was modified and 42% when diet and exercise were combined.

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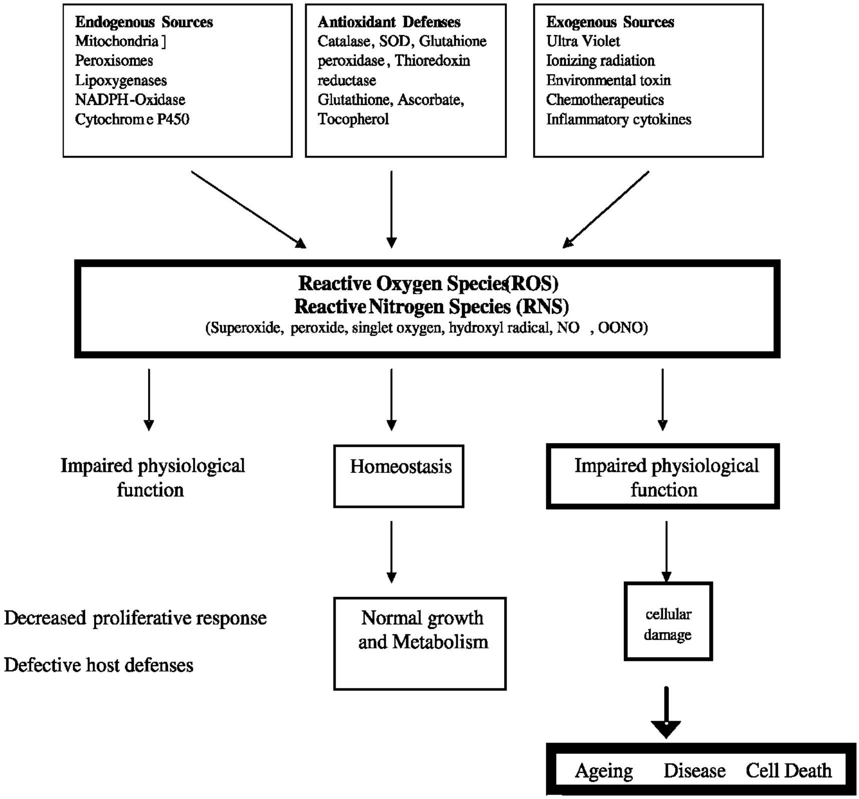
Endogenous Sources
Mitochondria]
Peroxisomes
Lipoxygenases
NADPH-Oxidase
Cytochrome P450
Antioxidant Defenses
Catalase, SOD, Glutahione peroxidase, Thioredoxin reductase
Glutathione, Ascorbate, Tocopherol
Exogenous Sources
Ultra Violet
Ionizing radiation
Environmental toxin
Chemotherapeutics
Inflammatory cytokines
Reactive Oxygen Species(ROS)
Reactive Nitrogen Species (RNS)
(Superoxide, peroxide, singlet oxygen, hydroxyl radical, NO , OONO)
Impaired physiological function
Homeostasis
Impaired physiological function
Decreased proliferative response
Defective host defenses
Normal growth and Metabolism
cellular damage
Ageing
Disease
Cell Death

Figure 3. Reactive oxygen species and cellular homeostasis. (Adapted from Shetty et al., 2008). (24).

Excessive α-amylase inhibition can lead to undigested starch in the colon and consequent stomach distention and discomfort (*51*). Recent studies have shown that phenolic compounds can play an important role in mediating α-amylase and α-glucosidase inhibition and therefore contributing to the management of type 2 diabetes (*52*). Thus, natural αamylase and αglucosidase inhibitors from fruits and vegetables could offer a good strategy to control the post-prandial hyperglycemia and provide benefits without the side effects present in the most available drugs such as abdominal distention, flatulence and possibly diarrhea (*53–60*). Phenolic phytochemicals have also been found to have potential in the management of oxidative stress linked chronic diseases like diabetes, cancer and cardiovascular disease (*6*, *61–64*).

Ford and Mokdad (*65*) reported a significant reduction in risk of type 2 diabetes among female participants consuming five or more servings of fruits and vegetables per day compared with those consuming none. Fogli-Cawley *et al.* (*66*) studied the association between The 2005 Dietary Guidelines for Americans (DGA) and the risk of the metabolic syndrome. The authors reported that the

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consumption of a diet following the DGA recommendations led to lower levels of many risks for metabolic syndrome along with reduced prevalence.

The beneficial effects of consumption of coffee by type 2 diabetic individuals have been reported (*67*, *68*) especially due to the presence of chlorogenic acid. The possible explanation is that chlorogenic acid might slow the carbohydrate absorption by inhibiting the glucose transport and exhibit a similar mechanism of action to the pharmaceutical drug (acarbose) used in the treatment of type 2 diabetes (*69–72*).

It has been reported that individuals affected by type 2 diabetes have associated risk factors, including hypertension, dyslipidemia and obesity (*73*). The renin-angiotensin system is important for regulating the blood pressure and water and electrolyte homeostasis (*74*). Angiotensin I-converting enzyme (ACE) is an important enzyme involved in maintaining vascular tension by two different reactions which it catalyzes: conversion of the inactive angiotensin I into a powerful vasoconstrictor and salt-retaining, angiotensin II, and inactivation of the vasodilator bradykinin, which is conducive to lowering blood pressure (*75*). Inhibition of this enzyme is considered a useful therapy in the control of blood pressure in hypertensive patients, which is a macro vascular complication of type 2 diabetes.

Suzuki *et al.* (*76*) reported an ACE inhibitory activity of chlorogenic acid in hypertensive rats with an improvement on the vasodilation. Suzuki *et al.* (*77*) reported the potential positive effect on hypertensive rats after administration of coffee. Also, water soluble extracts of green coffee bean, whose main component was chlorogenic acid, lowered blood pressure in mildly hypertensive patients (*78*). Edwards *et al.* (*79*) reported that the quercetin supplementation reduced the blood pressure in hypertensive subjects. These authors suggested that there is a potential for the use of this phenolic compound as adjunct therapy in diet / lifestyle interventions in the control of blood pressure in hypertensive individuals. Also, Nakamura *et al.* (*80*) reported positive effect in the vasorelaxation of rat aorta after administration of a black currant concentrate. The consumption of a high-flavonoid sweet juice (hybrid between grapefruit and pummelo) had significant beneficial effects on the reduction of blood pressure when compared to a low-flavonoid sweet juice (*81*). However, Nettleton *et al.* (*82*) reported that the ingestion of flavonoids and flavonoid-rich foods was not associated with reduction in the risk of type 2 diabetes in postmenopausal women. Only the regular consumption of red wine among these women was associated with decrease in the incidence of type 2 diabetes when compared to women that do not consume red wine.

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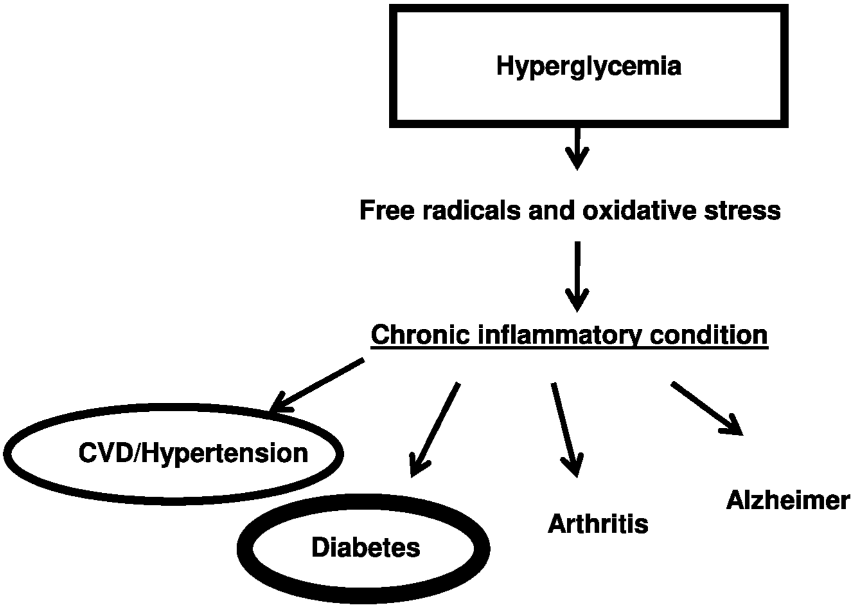
Hyperglycemia
Free radicals and oxidative stress
Chronic inflammatory condition
CVD/Hypertension
Diabetes
Arthritis
Alzheimer

Figure 4. Oxidative stress related hyperglycemia and complications. (Adapted from Shetty et al., 2008). (24).

Montonen *et al.* (*83*) suggested that the risk of developing type 2 diabetes could be reduced by changing dietary patterns and especially higher consumption of fruits and vegetables. Nothlings *et al.* (*38*) reported that the consumption of vegetables, legumes and fruits was significantly associated with reduced risks of all-cause mortality, not including cancer, in a European diabetic population. Also, these authors suggested that diabetic patients could benefit from a diet rich in fruits and vegetables. Miller *et al.* (*84*) reported that during an intervention in adults with type 2 diabetes, the participants selected more servings of whole fruits and nonfat dairy products and fewer servings of tomato-based products and vegetable fats, indicating that education and availability of information could help consumers make greater dietary changes.

Health Benefits of Berry Phenolics for Potential Type 2 Diabetes Management

As discussed previously αglucosidase and αamylase are well known enzymes in the management of hyperglycemia linked to type 2 diabetes (*85*). Many foods and herbal extracts have been reported as having positive effect in the diabetic glycemic control using traditional medicine (*86*, *87*). Khan *et al.* (*56*) studied common culinary herbs and spices and they observed improvement in the glucose metabolism was apparently due to phenolic compounds of the extracts. Botanical products can improve glucose metabolism and the overall condition of patients

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with type 2 diabetes not only by direct hypoglycemic effects but also by improving lipid metabolism, antioxidant status and capillary function (*88*).

In addition, many studies have reported the beneficial effects on hypertension by polyphenol-rich extracts not only by retarding the development of hypertension but also by normalizing the blood pressure (*89–91*). In this context, some foods and herbs could have the potential to treat hypertension, especially for patients with borderline to mild high blood pressure (*92*).

Berries are considered good sources of phenolic compounds such as flavonoids and phenolic acids. Besides this, berry fruits are commonly consumed not only as fresh but also as derived products such as canned fruits, yogurts, juices and jams. Many studies have reported the benefits of berry consumption against several types of human cancers (*39*, *93*), age-related neurodegenerative diseases (*31*) and metabolic syndrome (*94*).

McDougall and Stewart (*95*) reported that the polyphenols components of berries inhibit α-glucosidase and α-amylase enzymes, resulting in reduced blood glucose levels after starch-rich meals. McDougall *et al.* (*55*) studied the potential inhibitory activity of strawberries, blueberries, raspberries and black currants on α-glucosidase and α-amylase enzymes. These authors reported that strawberries and raspberries had the highest α-amylase inhibitory activity and blueberries and blackcurrants had the highest α-glucosidase inhibitory activity. According to Knekt *et al.* (*96*) and Song *et al.* (*97*) reduced risk for type 2 diabetes was associated with high consumption of apples and berries and a rich diet in fruits and vegetables.

McDougall *et al.* (*55*) studied high anthocyanin-containing fruits extracts from blueberry, currant, raspberry, and strawberry and they observed good αglucosidase inhibition by these extracts. However, Cheplick *et al.* (*98*) reported high α-glucosidase inhibitory activity for a yellow raspberry cultivar among red, black and yellow raspberries, suggesting that the α-glucosidase may be influenced more by specific anthocyanins rather than the actual amount of the overall total plant phenolics. Cheplick *et al.* (*98*) suggested that αamylase inhibitory activity in different raspberry cultivars might be due to some specific phenolics since many fruits including strawberries, raspberries, and grapes are known to contain high levels of soluble tannins and these fruits have α-amylase inhibitory properties (*55*).

Wilson *et al.* (*99*) suggested that cranberry juice could represent an attractive mean for increasing fruit intake and simultaneously affording positive health benefits. Apostolidis *et al.* (*100*) reported that cranberry-enriched cheese had the highest α-glucosidase and α-amylase inhibitory activities among herb, fruit and fungal-enriched cheeses by *in vitro* assays. Chambers and Camire (*101*) evaluated the ingestion of capsules filled with cranberry juice concentrate by adults with type 2 diabetes for 12 weeks. No significant difference in the blood glucose levels was observed. However these authors suggested that more concentrated products might have benefits since the commercially available cranberry juice cocktails contain only 27-31% cranberry juice.

Pinto *et al.* (*102*) studied the potential effects on the *in vitro* inhibition of αamylase and αglucosidase enzymes from different Brazilian strawberry cultivars. These authors reported that strawberries had high αglucosidase and low αamylase inhibitory activities, suggesting these fruits as good sources for potential management of hyperglycemia linked to type 2 diabetes as a part of an overall diet. Apostolidis *et al.* (*103*) reported that the combinations of cranberry with oregano which had higher rosmarinic acid content contributed to the high antioxidant activity and total phenolic content in the extracts, suggesting potential relevance for type 2 diabetes and related hypertension.

The dietary management of hyperglycemia linked to type 2 diabetes through foods that have high α-glucosidase and moderate α-amylase inhibition has been suggested (*104*). This is due to the fact that excessive α-amylase inhibition can lead to undigested starch in the colon and consequent stomach distention and discomfort (*51*). Therefore, berry fruits with high α-glucosidase and low α-amylase inhibitory activities could be considered as a good potential candidate as a part of an overall dietary design to manage early stages of hyperglycemia linked to type 2 diabetes.

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Summary

In summary, there are evidences that a diet rich in fruits and vegetables containing high levels of antioxidant compounds could potentially reduce the risk of certain chronic diseases such as type 2 diabetes and related micro and mascro vascular complications. Therefore within this context phenolic antioxidants from berries have excellent potential for managing type 2 diabetes through control of hyperglycemia and its marcovascular complications such as hypertension and microvascular complications linked to cellular oxidative breakdown. The considerable differences in the content and profile of phenolic compounds along with functionality among berry fruits can be exploited for designing consistent functional benefits for management of these chronic disease states linked to metabolic syndrome. Specifically, berries could be selected towards the contribution as a part of an overall healthy diet for the management of post-prandial hyperglycemia through their capacity to inhibit α-glucosidase concurrently with low inhibition of α-amylase likely resulting in less undesirable side effects. Further within the same profile bioactive phenolics also have the potential to combat hypertension and cellular oxidative breakdown linked macro and micro vascular complications, respectively.

References

1. Randhir, R.; Lin, Y-T.; Shetty, K. Stimulation of phenolics, antioxidant, and antimicrobial activities in dark germinated mung bean (*Vigna radiata*) sprouts in response to peptide and phytochemical elicitors. *Process Biochem.* **2004**, *39*, 637–646.
2. Bravo, L. Phenolic phytochemicals: Chemistry, dietary sources, metabolism, and nutritional significance. *Nutr. Rev.* **1998**, *56*, 317–333.

3. Mann, J. *Secondary Metabolism*; Oxford Chemistry Series; Clarendon Press: Oxford, 1978.
4. Vattem, D. A.; Randhir, R.; Shetty, K. Cranberry phenolics-mediated antioxidant enzyme response in oxidatively stressed porcine muscle. *Process Biochem.* **2005**, *40*, 2225–2238.
5. Chugh, L. K.; Sawhney, S. K. Effects of cadmium on activities of some enzymes of glycolysis and pentose phosphate pathway in pea. *Biol. Plant.* **1999**, *42*, 401–407.
6. Shetty, K.; Wahlqvist, M. L. A model for the role of the proline-linked pentose-phosphate pathway in phenolic phytochemical bio-synthesis and mechanism of action for human health and environmental applications. *Asia Pac. J. Clin. Nutr.* **2004**, *13*, 1–24.
7. Shetty, P.; Atallah, M. T.; Shetty, K. Stimulation of total phenolics, L-DOPA, and antioxidant activity through proline-linked pentose phosphate pathway in response to proline and its analog in germinating fava beans (*Vicia faba*). *Process Biochem.* **2003**, *38*, 1707–1717.
8. Acamovic, T.; Brooker, J. D. Biochemistry of plant secondary metabolites and their effects in animals. *Proc. Nutr. Soc.* **2005**, *64*, 403–412.
9. Alasalvar, C.; Grigor, J. M.; Zhang, D.; Quantick, P. C.; Shahidi, F. Comparison of volatiles, phenolics, sugars, antioxidant vitamins, and sensory quality of different colored carrot varieties. *J. Agric. Food Chem.* **2001**, *49* (3), 1410–1416.
10. Edreva, A.; Velikova, V.; Tsonev, T.; Dagnon, S.; Gürel, A.; Aktaş, L.; Gesheva, E. Stress-protective role of secondary metabolites: Diversity of functions and mechanisms. *Gen. Appl. Plant Physiol.* **2008**, *34* (1-2), 67–78.
11. Parr, A.; Bolwell, G. P. Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenol content or profile. *J. Sci. Food Agric.* **2000**, *80*, 985–1012.
12. Tomás-Barberán, F. A.; Espín, J. C. Phenolic compounds and related enzymes as determinants of quality in fruits and vegetables. *J. Sci. Food Agric.* **2001**, *81*, 853–876.
13. Lovelock, C. E.; Clough, B. F.; Woodrow, I. E. Distribution and accumulation of ultraviolet-radiation-absorbing compounds in leaves of tropical mangroves. *Planta* **1992**, *188*, 143–154.
14. Chaves, N.; Escudero, J. C.; Gutierrez-Merino, C. Role of ecological variables in the seasonal variation of flavonoid content of *Cistus ladanifer*. *J. Chem. Ecol.* **1997**, *23*, 579–604.
15. Alonso-Amelot, M. E.; Oliveros, A.; Calcagano-Pisarelli, M. P. Phenolics and condensed tannins in relation to altitude in neotropical Pteridium spp. A field study in the Venezuelan Andes. *Biochem. Syst. Ecol.* **2004**, *32*, 969–981.
16. Sullivan, J. H.; Teramura, A. H.; Ziska, L. H. Variation in UV-B sensitivity in plants from a 3000 m elevational gradient in Hawaii. *Am. J. Bot.* **1992**, *79*, 737–743.
17. Prior, R. L.; Cao, G.; Martin, A.; Sofic, E.; McEwan, J.; O'Brien, C.; Lischner, N.; Ehlenfeldt, M.; Kalt, W.; Krewer, G.; Mainland, C. M.

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Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of Vaccinium species. *J. Agric. Food Chem.* **1998**, *46*, 2686–2693.

18. Deighton, N.; Brennan, R.; Finn, C.; Davies, H. V. Antioxidant properties of domesticated and wild Rubus species. *J. Sci. Food Agric.* **2000**, *80*, 1307–1313.
19. Pinto, M. S.; Lajolo, F. M.; Genovese, M. I. Bioactive compounds and quantification of total ellagic acid in strawberries (*Fragaria x ananassa* Duch.). *Food Chem.* **2008**, *107*, 1629–1635.
20. Hertog, M. G. L.; Hollmam, P. C. H.; Katan, M. B. Content of potentially anticarcinogenic flavonoids of tea infusion, wines, and fruit juices. *J. Agric. Food Chem.* **1993**, *41*, 1242–1246.
21. Heima, K. E.; Tagliaferroa, A. R.; Bobilya, D. J. Flavonoid antioxidants: Chemistry, metabolism, and structure-activity relationships. *J. Nutr. Bioch.* **2002**, *13* (10), 572–584.
22. Balasundram, N.; Sundram, K.; Samman, S. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chem.* **2006**, *99*, 191–203.
23. Pinto, M. S.; Lajolo, F. M.; Genovese, M. I. Bioactive compounds and antioxidant capacity of strawberry jams. *Plant Foods Hum. Nutr.* **2007**, *62*, 127–131.
24. Shetty, K.; Adyanthaya, I.; Kwon, Y.-I.; Apostolidis, E.; Min, B.-J.; Dawson, P. Postharvest enhancement of phenolic phytochemicals in apples for preservation and health benefits. In *Postharvest Biology and Technology of Fruits, Vegetables, and Flowers*; Paliyath G., Murr D., Handa A. K., Lurie S., Eds.; Wiley-Blackwell Publishing: Ames, IA, 2008; Chapter 16, pp 341−371.
25. Urso, M. L.; Clarkson, P. M. Oxidative stress, exercise, and antioxidant supplementation. *Toxicol.* **2003** (189), 41–54.
26. Halliwell, B. Effect of diet on cancer development: Is oxidative DNA damage a biomarker?. *Free Radical Biol. Med.* **2002**, *32*, 968–974.
27. Aviram, M. Macrophage foam cell formation during early atherogenesis is determined by the balance between pro-oxidants and antioxidants in arterial cells and blood lipoproteins. *Antioxid. Redox Signaling* **1999**, *1*, 585–594.
28. Eastwood, M.A. Interaction of dietary antioxidants *in vivo*: How fruit and vegetables prevent disease?. *Q. J. Med.* **1999**, *92*, 527–530.
29. Hollman, P. C. H.; Katan, M. B. Dietary flavonoids: Intake, health effects, and bioavailability. *Food Chem. Toxicol.* **1999**, *37*, 937–942.
30. Middleton, E., Kandaswami, C. The impact of plant flavonoids on mammalian biology: Implications for immunity, inflammation and cancer. In *The Flavonoids. Advances in Research Since 1986*; Harborne, J. B., Ed.; Chapman & Hall: London, 1994; pp 619−652.
31. Shukitt-Hale, B.; Lau, F. C.; Joseph, J. A. Berry fruit supplementation and the aging brain. *J. Agric. Food Chem.* **2008**, *56*, 636–41.
32. Wei, Z.; Lau, B. H. S. Garlic inhibits free radical generation and augments antioxidant enzyme activity in vascular endothelial cells. *Nutr. Res.* **1998**, *18*, 61–70.

33. Dembinska-Kiec, A.; Mykkänen, O.; Kiec-Wilk, B.; Mykkänen, H. Antioxidant phytochemicals against type 2 diabetes. *Br. J. Nutr.* **2008**, *99*, ES109–ES117.
34. Hung, H. C.; Joshipura, K. J.; Jiang, R.; Hu, F. B.; Hunter, D.; Smith-Warner, S. A.; Colditz, G. A.; Rosner, B.; Spiegelman, D.; Willett, W. C. Fruit and vegetable intake and risk of major chronic disease. *J. Natl. Cancer Inst.* **2004**, *96*, 1577–1584.
35. Costacou, T.; Mayer-Davis, E. J. Nutrition and prevention of type 2 diabetes. *Ann. Rev. Nutr.* **2003**, *23*, 147–170.
36. Esposito, K.; Nappo, F.; Marfella, R.; Giugliano, G.; Giugliano, F.; Ciotola, M.; Quagliaro, L.; Ceriello, A.; Giugliano, D. Inflammatory cytokine concentrations are acutely increased by hyperglycemia in humans: Role of oxidative stress. *Circulation* **2002**, *106*, 2067–2072.
37. Joshipura, K. J.; Hu, F. B.; Manson, J. E.; Stampfer, M. J.; Rimm, E. B.; Speizer, F. E.; Colditz, G.; Ascherio, A.; Rosner, B.; Spiegelman, D.; Willett, W. C. The effect of fruit and vegetable intake on risk for coronary heart disease. *Ann. Intern. Med.* **2001**, *132*, 1106–1114.
38. Nöthlings, U.; Schulze, M. B.; Weikert, C.; Boeing, H.; Van der Schouw, Y. T.; Bamia, C.; Benetou, V.; Lagiou, P.; Krogh, V.; Beulens, J. W. J.; Peeters, P. H. M.; Halkjær, J.; Tjønneland, A.; Tumino, R.; Panico, S.; Masala, G.; Clavel-Chapelon, F.; De Lauzon, B.; Boutron-Ruault, M. C.; Vercambre, M. N.; Kaaks, R.; Linseisen, J.; Overvad, K.; Arriola, L.; Ardanaz, E.; Gonzalez, C. A.; Tormo, M. J.; Bingham, S.; Khaw, K. T.; Key, T. J. A.; Vineis, P.; Riboli, E.; Ferrari, P.; Boffetta, P.; Bueno-de-Mesquita, H. B.; Van der A, D. L.; Berglund, G.; Wirfält, E.; Hallmans, G.; Johansson, I.; Lund, E.; Trichopoulo, A. Intake of vegetables, legumes, and fruit, and risk for all-cause, cardiovascular, and cancer mortality in a European diabetic population. *J. Nutr.* **2008**, *138*, 775–781.
39. Seeram, N. P. Berry fruits for cancer prevention: Current status and future prospects. *J. Agr. Food Chem.* **2008**, *56*, 630–635.
40. Espín, J. C.; García-Conesa, M. T.; Tomás-Barberán, F. A. Nutraceuticals: Facts and fiction. *Phytochemistry* **2007**, *68*, 2986–3008.
41. Wild, S.; Roglic, G.; Green, A.; Sicree, R.; King, H. Global prevalence of diabetes: Estimates for the year 2000 and projections for 2030. *Diabetes Care* **2004**, *27*, 1047–1053.
42. Daar, A. S.; Singer, P. A.; Persad, D. L.; Pramming, S. K.; Matthews, D. R.; Beaglehole, R.; Bernstein, A.; Borysiewicz, L. K.; Colagiuri, S.; Ganguly, N.; Glass, R. I.; Finegood, D. T.; Koplan, J.; Nabel, E. G.; Sarna, G.; Sarrafzadegan, N.; Smith, R.; Yach, D.; Bell, J. Grand challenges in chronic non-communicable diseases. *Nature* **2007**, *450*, 494–496.
43. Winer, N.; Sowers, J. R. Epidemiology of diabetes. *J. Clin. Pharmacol.* **2004**, *44*, 397–405.
44. King, H.; Aubert, R. E.; Herman, W. H. Global burden of diabetes, 1995-2025: Prevalence, numerical estimates, and projections. *Diabetes Care* **1998**, *21* (9), 1414–1431.
45. DeFronzo, R. A.; Bonadonna, R. C.; Ferrannini, E. Pathogenesis of NIDDM. A balanced overview. *Diabetes Care* **1992**, *15*, 318–368.

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): March 18, 2010 | doi: 10.1021/bk-2010-1035.ch008

46. Baynes, J. W.; Thorpe, S. R. Perspectives in diabetes. Role of oxidative stress in diabetic complications. A new perspective on an old paradigm. *Diabetes* **1999**, *48*, 1–9.
47. Pickup, J. C. Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes. *Diabetes Care* **2004**, *27*, 813–823.
48. García-Alonso, J.; Rosa, G.; Vidal-Guevarab, M. L.; Periago, M. J. Acute intake of phenolic-rich juice improves antioxidant status in healthy subjects. *Nutr. Res.* **2006**, *26*, 330–339.
49. Ford, E. S.; Giles, W. H.; Mokdad, A. H. Increasing prevalence of the metabolic syndrome among U.S. adults. *Diabetes Care* **2004**, *27*, 2444–2449.
50. Pan, X. R.; Li, G. W.; Hu, Y. H.; Wang, J. X.; Yang, W. Y.; An, Z. X.; Hu, Z. X.; Lin, J.; Xiao, J. Z.; Cao, H. B.; Liu, P. A.; Jiang, X. G.; Jiang, Y. Y.; Wang, J. P.; Zheng, H.; Zhang, H.; Bennett, P. H.; Howard, B. V. Effects of diet and exercise in preventing NIDDM in people with impaired glucose tolerance. The Da Qing IGT and Diabetes Study. *Diabetes Care* **1997**, *20*, 537–544.
51. Puls, W.; Keup, U.; Krause, H.; Thomas, P. G.; Hoffmeister, F. Glucosidase inhibition: A new approach to the treatment of diabetes, obesity, and hyperlipoproteinemia. *Naturwissenschaften* **1977**, *64*, 536–537.
52. McCue, P.; Shetty, K. Inhibitory effects of rosmarinic acid extracts on porcine pancreatic amylase and implications for health. *Asia Pac. J. Clin. Nutr.* **2004**, *13*, 101–106.
53. Matsui, T.; Ueda, T.; Oki, T.; Sugita, K.; Terahara, N.; Matsumoto, K. α-Glucosidase inhibitory action of natural acylated anthocyanins. 1. Survey of natural pigments with potent inhibitory activity. *J. Agric. Food Chem.* **2001**, *49*, 1948–1951.
54. Kwon, Y. I.; Vattem, D. V.; Shetty, K. Evaluation of clonal herbs of Lamiaceae species for management of diabetes and hypertension. *Asia Pac. J. Clin. Nutr.* **2006**, *15*, 107–118.
55. McDougall, G. J.; Shpiro, F.; Doboson, P.; Smith, P.; Black, A.; Stewart, D. Different polyphenolic compounds of soft fruits inhibit α-amylase and α-glucosidase. *J. Agric. Food Chem.* **2005**, *53*, 2760–2766.
56. Khan, A.; Bryden, N. A.; Polansky, M. M.; Anderson, R. A. Insulin potentiating factor and chromium content of selected foods and spices. *Biol. Trace Elem. Res.* **1990**, *24*, 183–188.
57. Kotowaroo, M. I.; Mahomoodally, M. F.; Gurib-Fakim, A.; Subratty, A. H. Screening of traditional antidiabetic medicinal plants of Mauritius for possible α-amylase inhibitory effects in vitro. *Phytother. Res.* **2006**, *20*, 228–231.
58. Gao, H.; Huang, Y. N.; Xu, P. Y.; Kawabata, J. Inhibitory effect on α-glucosidase by the fruits of *Terminalia chebula* Retz. *Food Chem.* **2007**, *105*, 628–634.
59. Bhandari, M. R.; Jong-Anurakkun, N.; Hong, G.; Kawabata, J. α-Glucosidase and α-amylase inhibitory activities of Nepalese medicinal herb Pakhanbhed (*Bergenia ciliata*, Haw.). *Food Chem.* **2008**, *106*, 247–252.

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): March 18, 2010 | doi: 10.1021/bk-2010-1035.ch008

60. Ali, H.; Houghton, P. J.; Soumyanath, A. α-Amylase inhibitory activity of some Malaysian plants used to treat diabetes; with particular reference to *Phyllanthus amarus*. *J. Ethnopharmacol.* **2006**, *107*, 449–455.
61. Shetty, K. Biotechnology to harness the benefits of dietary phenolics; Focus on Lamiaceae. *Asia Pac. J. Clin. Nutr.* **1997**, *6*, 162–171.
62. Shetty, K.; Labbe, R. L. Food-borne pathogens, health and role dietary phytochemicals. *Asia Pac. J. Clin. Nutr.* **1998**, *7*, 270–276.
63. Shetty, K. Phytochemicals: Biotechnology of phenolic phytochemicals for food preservatives and functional food applications. In *Wiley Encyclopedia of Food Science and Technology*, 2nd ed.; Francis, F. J., Ed.; Wiley Publishers: New York, 1999; pp 1901−1909.
64. Shetty, K. Biosynthesis of rosmarinic acid and applications in medicine. *J. Herbs, Spices, Med. Plants* **2001**, *8*, 161–181.
65. Ford, E. S.; Mokdad, A. H. Fruit and vegetable consumption and diabetes mellitus incidence among U.S. adults. *Prev. Med.* **2001**, *32*, 33–39.
66. Fogli-Cawley, J. J.; Dwyer, J. T.; Saltzman, E.; McCullough, M. L.; Troy, L. M.; Meigs, J. B.; Jacques, P. F. The 2005 Dietary Guidelines for Americans and risk of the metabolic syndrome. *Am. J. Clin. Nutr.* **2007**, *86* (4), 1193–1201.
67. Greenberg, J. A.; Boozer, C. N.; Geliebter, A. Coffee, diabetes, and weight control. *Am. J. Clin. Nutr.* **2006**, *84*, 682–693.
68. McCarty, M. F. Nutraceutical resources for diabetes preventionsAn update. *Med. Hypotheses* **2005**, *64*, 151–158.
69. Welsch, C. A.; Lachance, P. A.; Wasserman, B. P. Dietary phenolic compounds: inhibition of Na+-dependent d-glucose uptake in rat intestinal brush border membrane vesicles. *J. Nutr.* **1989**, *119*, 1698–1704.
70. Johnston, K. L.; Clifford, M. N.; Morgan, L. M. Coffee acutely modifies gastrointestinal hormone secretion and glucose tolerance in humans: Glycemic effects of chlorogenic acid and caffeine. *Am. J. Clin. Nutr.* **2003**, *78*, 728–733.
71. Feinberg, L. J.; Sandberg, H.; De Castro, O.; Bellet, S. Effects of coffee ingestion on oral glucose tolerance curves in normal human subjects. *Metabolism* **1968**, *17*, 916–922.
72. Bassoli, B. K.; Cassolla, P.; Borba-Murad, G. R.; Constantin, J.; Salgueiro-Pagadigorria, C. L.; Bazotte, R. B.; Silva, R. S. S. F.; Souza, H. M. Chlorogenic acid reduces the plasma glucose peak in the oral glucose tolerance test: Effects on hepatic glucose release and glycaemia. *Cell Biochem. Funct.* **2008**, *26*, 320–328.
73. Harris, M. I. Summary. In *Diabetes in America*, 2nd ed.; NIH Publication Number 95-1468; U.S. Government Printing Office: Washington, DC, 1995; pp 1–14.
74. Guyton, A. C.; Hall, J. E. *Textbook of Medical Physiology*, 10th ed.; W. B. Saunders Company: Philadelphia, 2000; issue 19.
75. Johnston , C. I.; Franz, V. Renin-angiotensin system: A dual tissue and hormonal system for cardiovascular control. *J. Hypertens.* **1992**, *10*, 13–26.

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): March 18, 2010 | doi: 10.1021/bk-2010-1035.ch008

76. Suzuki, A.; Kagawa, D.; Fujii, A.; Ochiai, R.; Tokimitsu, I.; Saito, I. Short and long-term effects of ferulic acid on blood pressure in spontaneously hypertensive rats. *Am. J. Hypertens.* **2002**, *15*, 351–357.
77. Suzuki, A.; Fujii, A.; Jokura, H.; Tokimitsu, I.; Hase, T.; Saito, I. Hydroxyhydroquinone interferes with the chlorogenic acid-induced restoration of endothelial function in spontaneously hypertensive rats. *Am. J. Hypertens.* **2008**, *21*, 23–27.
78. Kozuma, K.; Tsuchiya, S.; Kohori, J.; Hase, T.; Tokimitsu, I. Antihypertensive effect of green coffee bean extract on mildly hypertensive subjects. *Hypertens. Res.* **2005**, *28*, 711–718.
79. Edwards, R. L.; Lyon, T.; Litwin, S. E.; Rabovsky, A.; Symons, J. D.; Jalili, T. Quercetin reduces blood pressure in hypertensive subjects. *J. Nutr.* **2007**, *137*, 2405–2411.
80. Nakamura, Y.; Matsumoto, H.; Todoki, K. Endothelium-dependent vasorelaxation induced by black currant concentrate in rat thoracic aorta. *Jpn. J. Pharmacol.* **2002**, *89*, 29–35.
81. Reshefa, N.; Hayarib, Y.; Gorenb, C.; Boazc, M.; Madarb, Z.; Knoblera, H. Antihypertensive effect of sweetie fruit in patients with stage I hypertension. *Am. J. Hypertens.* **2005**, *18* (10), 1360–1363.
82. Nettleton, J. A.; Harnack, L. J.; Scrafford, C. G.; Mink, P. J.; Barraj, L. M.; Jacobs, D. R. Dietary flavonoids and flavonoid-rich foods are not associated with risk of type 2 diabetes in postmenopausal women. *J. Nutr.* **2006**, *136*, 3039–3045.
83. Montonen, J.; Knekt, P.; Härkänen, T.; Järvinen, R.; Heliövaara, M.; Aromaa, A.; Reunanen, A. Dietary patterns and the incidence of type 2 diabetes. *Am. J. Epidemiol.* **2005**, *161* (3), 219–227.
84. Miller, C. K.; Gutshcall, M. D.; Mitchell, D. C. Change in food choices following a glycemic load intervention in adults with type 2 diabetes. *J. Am. Diet. Assoc.* **2009**, *109*, 319–324.
85. Bailey, C. J. New approaches to the pharmacotherapy of diabetes. In *Textbook of Diabetes*, 3rd ed.; Pickup J. C., William, G., Eds.; Blackwell Science Ltd.: Oxford, UK, 2003; Vol. 2, pp 73.1–73.21.
86. Yeh, G. Y.; Eisenberg, D. M.; Kaptchuk, T. J.; Phillips, R. S. Systematic review of herbs and dietary supplements for glycemic control in diabetes. *Diabetes Care* **2003**, *26*, 1277–1294.
87. Jia, W.; Gao, W.; Tang, L. Antidiabetic herbal drugs officially approved in China. *Phytother. Res.* **2003**, *17*, 1127–1134.
88. Broadhurst, C. L. Nutrition and non-insulin dependent diabetes from an anthropological perspective. *Altern. Med. Rev.* **1997**, *2*, 378–399.
89. Taubert, D.; Berkels, R.; Roesen, R.; Klaus, W. Chocolate and blood pressure in elderly individuals with isolated systolic hypertension. *JAMA* **2003**, *290*, 1029–1030.
90. Negishi, H.; Xu, J. W.; Ikeda, K.; Njelekela, M.; Nara, Y.; Yamori, Y. Black and green tea polyphenols attenuate blood pressure increases in stroke-prone spontaneously hypertensive rats. *J. Nutr.* **2004**, *134*, 38–42.
91. Appel, L. J.; Moore, T. J.; Obarzanek, E.; Vollmer, W. M.; Svetkey, L. P.; Sacks, F. M.; Bray, G. A.; Vogt, T. M.; Cutler, J. A.; Windhauser, M. M.;

Downloaded by COLUMBIA UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): March 18, 2010 | doi: 10.1021/bk-2010-1035.ch008

Lin, P. H.; Karanja, N. A clinical trial of the effects of dietary patterns on blood pressure. DASH Collaborative Research Group. *N. Engl. J. Med.* **1997**, *336*, 1117–1124.

92. Chen, Z. Y.; Peng, C.; Jiao, R.; Wong, Y. M.; Yang, N.; Huang, Y. Anti-hypertensive nutraceuticals and functional foods. *J. Agric. Food. Chem.* **2009**, *57*, 4485–4499.
93. Juranic, Z.; Zizak, Z. Biological activities of berries: From antioxidant activity to anti-cancer effects. *BioFactors* **2005**, *23*, 207–211.
94. Tsuda, T. Regulation of adipocyte function by anthocyanins; possibility of preventing the metabolic syndrome. *J. Agr. Food Chem.* **2008**, *56*, 642–646.
95. McDougall, G. J.; Stewart, D. The inhibitory activity effects of berry polyphenols on digestive enzymes. *BioFactors* **2005**, *23*, 189–195.
96. Knekt, P.; Kumpulainen, J.; Jarvinen, R.; Rissanen, H.; Heliovaara, M.; Reunanen, A.; Hakulinen, T.; Aromaa, A. Flavonoid intake and risk of chronic diseases. *Am. J. Clin. Nutr.* **2002**, *76*, 560–568.
97. Song, Y.; Manson, J. E.; Buring, J. E.; Sesso, H. D.; Liu, S. Associations of dietary flavonoids with risk of type 2 diabetes, and markers of insulin resistance and systemic inflammation in women: A prospective study and crosssectional analysis. *J. Am. Coll. Nutr.* **2005**, *24*, 376–384.
98. Cheplick, S.; Kwon, Y.; Bhowmik, P.; Shetty, K. Clonal variation in raspberry fruit phenolics and relevance for diabetes and hypertension management. *J. Food Biochem.* **2007**, *31*, 656–679.
99. Wilson, T.; Singh, A. P.; Vorsa, N.; Goettl, C. D.; Kittleson, K. M.; Roe, C. M.; Kastello, G. M.; Ragsdale, F. R. Human glycemic response and phenolic content of unsweetened cranberry juice. *J. Med. Food* **2008**, *11*, 46–54.
100. Apostolidis, E.; Kwon, Y. I.; Shetty, K. Inhibitory potential of herb, fruit, and fungal-enriched cheese against key enzymes linked to type 2 diabetes and hypertension. *Inn. Food Sci. Emer. Technol.* **2007**, *8*, 46–54.
101. Chambers, B. K.; Camire, M. E. Can cranberry supplementation benefit adults with type 2 diabetes?. *Diabetes Care* **2003**, *26*, 2695–2696.
102. Pinto, M. S.; Kwon, Y. I.; Apostolidis, E.; Lajolo, F. M.; Genovese, M. I.; Shetty, K. Functionality of bioactive compounds in brazilian strawberry (*Fragaria x ananassa* Duch.) cultivars: Evaluation of hyperglycemia and hypertension potential using in vitro models. *J. Agric. Food Chem.* **2008**, *56*, 4386–4382.
103. Apostolidis, E.; Kwon, Y. I.; Shetty, K. Potential of cranberry-based herbal synergies for diabetes and hypertension management. *Asia Pac. J. Clin. Nutr.* **2006**, *15*, 433–441.
104. Kwon, Y. I.; Vattem, D. V.; Shetty, K. Evaluation of clonal herbs of Lamiaceae species for management of diabetes and hypertension. *Asia Pac. J. Clin. Nutr.* **2006**, *15*, 107–118.

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Chapter 9

Vasoactive and Vasoprotective Antioxidant Properties of Anthocyanin-Enriched Berry Extracts

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Cardiovascular disease is associated with impaired arterial function that is strongly related to both impairment of the arterial endothelial nitric oxide system and exposure of arteries to excessive concentrations of reactive oxygen species. Agents that can stimulate the arterial NO system and/or attenuate vascular damage from oxygen radicals have the potential to produce beneficial effects in cardiovascular disease. Recent vascular experiments have shown that anthocyanin-enriched berry extracts both stimulate coronary arterial NO systems and protect arteries from damage due to oxygen radicals.

Arteries and Cardiovascular Disease

Cardiovascular disease is the number one cause of mortality and morbidity in the United States. Currently, as many men and women die of cardiovascular disease in the United States as do from all other forms of mortality combined (*1*). Although most individuals think of cardiovascular disease as a dysfunction of the heart, most all cardiovascular disease is either caused by or has a major contribution from problems associated with the arteries of the cardiovascular system. Arteries form part of the "vascular" component of the cardiovascular system. They are tubular conduits that transport oxygenated blood to the organs of the body as it is received through the pumping action of the left side of the heart. These blood vessels, however, are not simple passive tubes that simply convey fluid from one point to another. Rather, arteries are dynamic organs

In Flavor and Health Benefits of Small Fruits; Qian, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

capable of changing the cross-sectional area of their lumen and therefore their resistance to blood flow. This simple, dynamic, physical alteration is used in the body to control organ blood flow, intra-organ blood flow distribution and arterial blood pressure.

Arteries are capable of changing their cross sectional artery by virtue of contracting or relaxing circular layers of smooth muscle contained within the center (media) of their walls. Contraction of arterial smooth muscle (vasoconstriction) reduces the cross section of the artery lumen and increases its resistance to flow whereas relaxation of this muscle (vasodilation or vasorelaxation) has the opposite effect. Thus, in a given organ, vasoconstriction reduces blood flow and oxygen delivery whereas vasodilation allows blood flow and oxygen delivery to increase. When vasoconstriction occurs simultaneously in many systemic organs at the same time, or to a significant extent in large organ systems such as skeletal muscle and the digestive tract, the resulting increase in whole body peripheral vascular resistance results in an increase in arterial blood pressure. Conversely, arterial pressure can be lowered through significant systemic arterial vasodilation which lowers whole body peripheral vascular resistance. These effects are hydraulic analogies of Ohm's law.

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The Role of the Arterial Endothelium and Nitric Oxide in Vascular Function

Arterial vasoconstriction or vasodilation ,which collectively is often referred to as *vascular reactivity*, is altered by literally scores of chemical and physical factors. Some of these factors occur naturally within the body and are involved with regulation of organ blood flow and arterial blood pressure. Arteries also respond to pharmaceutical agents specifically designed to alter arterial reactivity as well as abnormal vasoactive stimuli associated with many diseases.

The key factor, however, regulating the level of contraction on arteries is the radical nitric oxide (NO) which is released intrinsically by the single layer of endothelial cells lining the lumen of all arteries and veins (see Figure 1).

Nitric oxide (NO) produced by arterial endothelium is not only a potent regulator of arterial contraction but also impacts other important vascular functions in both health and disease (*2*). The endothelium produces NO through the action of a constitutively expressed intracellular enzyme, nitric oxide synthase (eNOS), which catalyzes the conversion of the intracellular amino acid, L-arginine, to L-citrulline and NO. Nitric Oxide synthase is activated by a calcium:calmodulin complex, which in turn is formed whenever calcium enters the endothelial cells containing eNOS. Calcium influx occurs through membrane channels in the endotlhelium and is normally stimulated by the shear stress exerted on that tissue by local blood flow. However, numerous receptor mediated dilator agents such as acetylcholine and histamine mediate vasorelaxation by activating this system. NOS can also be activated in endothelial cells or arteries laboratory experiments by exposing those tissues to calcium ionophore drugs such as A23187. NO is highly diffusible and lipophylic which enables it to easily diffuse from within arterial endothelial cells into the underlying arterial smooth muscle. Once there NO activates guanylate cyclase which catalyses the formation of cGMP from GTP. cGMP is a smooth muscle second messenger that stimulates a protein kinase messenger cascade which results in eventual relaxation of the arterial muscle.

NO is released tonically in all arteries and is a potent vasodilator. It helps prevent severe vasoconstriction which could otherwise throttle tissue blood flow and oxygen supply. By providing a constant background of vasorelaxation stimuli to arteries it also helps keep peripheral vascular resistance low enough to prevent hypertension (high blood pressure) which can otherwise lead to heart failure and heart attack.

Although its vasodilatory properties were the first discovered cardiovascular effects of NO, this simple radical has many other beneficial vascular properties. NO is a strong inhibitor of platelet aggregation (*2*), which is an initial step in forming blood clots. Therefore, NO helps prevent blood clot formation in arteries. NO inhibits interaction of neutrophils with the arterial endothelium which is an initial response to arterial injury associated with the development of atherosclerosis (*2*). This disease, associated with the high blood cholesterol levels and cholesterol laden plaques in the artery wall, is actually an inflammatory process that also stimulates abnormal inward growth of smooth muscle cells into the lumen of arteries. In healthy arteries such vascular muscle cell growth is normally inhibited by NO.

NO is known to attenuate ischemia reperfusion injury (*2*). When tissues are deprived of adequate blood flow the resulting ischemia, or inadequate oxygen supply, results in cell damage and eventual cell death. Unfortunately, restoration of tissue blood flow following an episode of ischemia in any tissue, especially the heart, actually initially exacerbates the ischemic injury. However, it has been shown that this reperfusion injury can be reduced by NO.

The Role of NO and O_2^- in Vascular Disease

In spite of all its beneficial vascular effects, the endothelial NO system has been shown to be impaired in all forms of cardiovascular disease. This includes hypertension, atherosclerosis, heart failure, myocaridial ischemia and the ascular complication associated with diabetes mellitus (*3*). Impairmnet of this system is considered a key factor in the morbidity and mortality associated with the development and progression of cardiovascular disease. Malfunction of the endothelial NO system in cardiovascular disease is likely related in part to a key interaction between NO and another oxygen radical, superoxide, which is produced in and around arteries. Superoxide is a natural byproduct of the 4 step reduction of O_2 to H_2O during oxidative metabolism in cells. Superoxide can be considered the functional antithesis of NO in term of its vascular properties. It is vasoconstrictive and therefore considered a vasospastic and hypertensive agent. Superoxide is pro-thrombotic and pro-atherogenic. Superoxide and any additional reactive oxygen species it forms play a key role in arterial injury and inflammation in atherosclerosis, diabetes and ischemia reperfusion injury. It also directly and

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indirectly damages the arterial endothelium responsible for the normal production of NO in arteries. Finally, superoxide rapidly reacts with NO and can markedly reduce the bioavailability of NO to the underlying vascular smooth muscle. However, these two radicals may be considered to be mutually inactivating (see Figure 1); although it is correct to say that excess superoxide production may markedly reduce thel effects of NO in the cardiovascular system it also true that NO can protect tissues from oxidant injury by quenching superoxide.

Unfortunately, not only is the NO system itself impaired in all forms of cardiovascular disease but production of reactive oxygen species in and around arteries is markedly increased in these diseases as well (*4–7*). Thus, in cardiovascular disease the functional balance between these two radicals is tipped in the direction of rendering an individual prone to hypertension, accelerated atherosclerosis, blood clot formation and general vascular damage. Any of these factors can result in markedly increased morbidity and probability of death in an individual.

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Functional Targets for Novel Botanical Compounds Proposed for the Treatment or Prevention of Cardiovascular Disease

The interplay between the beneficial effects of NO and the detrimental effects of O_2^- on the vascular system suggests a prime target for intervention by any novel agent that could potentially be employed to attenuate the vascular pathologies associated with cardiovascular disease. It is clear that any agent capable of enhancing the arterial NO system and/or attenuating the detrimental vascular effects of O_2^- has the potential to produce a significant beneficial effect in cardiovascular disease. In terms of novel botanicals or agents contained in food products, recent studies suggest that such potential vasoprotective agents may be found among the flavonoids and polyphenolic compounds contained in many fruits. Polyphenolic compounds, especially anthocyanins which make up the red, blue and purple pigments of many fruits (*8*), are known to be potent oxygen radical scavengers (*9*). However, the potential of such fruits or their components to affect vascular function, especially in terms of protection from oxygen radical assault, has just started to be investigated by the biomedical community. Such investigations are important in the sense that although diets rich in fruits and vegetables have long been known to be associated with reductions in the incidence of cardiovascular disease, studies seeking to link specific nutritional components contained in fruits and vegetables, such as vitamins C, E and beta-carotene, have failed to show cardiovascular benefits (*10*, *11*). It is therefore possible that other components of fruits and vegetables, previously disregarded as non-nutritional, may in fact be the real source of cardioprotective properties of these foods in the diet.

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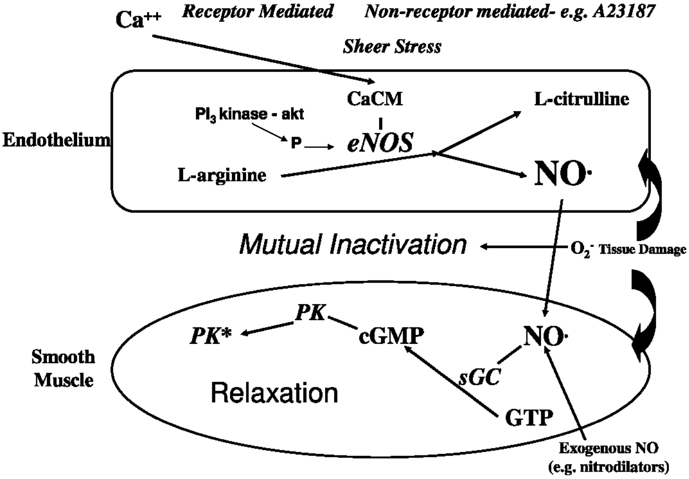
Ca++
Receptor Mediated
Non-receptor mediated- e.g. A23187
Sheer Stress
Endothelium
PI3 kinase - akt
P
CaCM
eNOS
L-arginine
L-citrulline
NO·
Mutual Inactivation
O2- Tissue Damage
Smooth Muscle
PK*
PK
cGMP
NO·
sGC
GTP
Relaxation
Exogenous NO
(e.g. nitrodilators)

Figure 1. A diagrammatic representation of the arterial endothelium vasodilator mechanism. CaCM = calcium:Calmodulin complex, P = Phosphophate, PI_3-Akt = PI_3-Akt protein phosphylation cascade for enhancing sensitivity of eNOS, sGC = soluble quanilate cyclase, PK = inactive cGMP dependent protein kinase, PK = active cGMP dependent protein kinase.*

Methods of Evaluation of Arterial Function

In Vivo vs *In Vitro* Analyses of Vascular Function

The effects of agents that can affect arterial function are typically studied using either *in vivo* and *in vitro* experimental designs. *In vivo* studies employ dietary consumption, subcutaneous implantation or IV injection of test substances in whole animals or human subjects and provide an opportunity to evaluate vascular functional alterations following long term exposure of the subject to the test agents. However, interpretation of *in vivo* studies is complicated if one wishes to determine whether a test agent has any direct effects on arterial function. It is always possible that any change in arterial function seen following exposure of the whole organism to a test agent is not due to direct effects of the agent on the artery itself but rather results from secondary effects brought about by alterations in other physiological or biochemical processes with the whole organism. *In vivo* experimental designs are further complicated by issues concerning the bioavailability of the test substance over timeas well by the fact that the concentration of the substance at any location and time in the body is affected by its metabolism and elimination by the organism.

Problems associated with analysis of arterial function in *in vivo* studies are controlled better with *in vitro* studies. *In vitro* studies often examine functions in

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isolated arterial segments in physiological solutions or utilize tissue culture and cell culture test platforms to examine the functions of arterial cell components. These methods allow assessment of arterial functions following direct exposure of the tissue to a test substance of known concentration in a controlled environment. Such assessment makes it easier to determine whether a novel agent itself can interact with arteries and alter vascular function.

Analyses of Vascular Function Using *In Vitro* Isometric Force Recordings of Isolated Arterial Segments

One of the long standing methods used to study artery physiology and pharmacology uses measurement of isometric arterial contraction and relaxation in isolated rings of arteries placed in controlled environments *in vitro* (*12*). This method is often referred to as *in vitro* arterial isometric force recording. In this method arteries from a given organ, such as the heart, brain or skeletal muscle are obtained from experimental animals, cleaned and cut into ring segments approximately 3-4 mm in width. These arterial rings are then suspended between supports and isometric force transducers in temperature controlled "artery baths" which contain oxygenated, pH controlled physiological solutions necessary for arterial viability. Arteries in such settings respond to physiological and pharmacological stimuli in an identical manner to that seen in the intact animal. Vasoconstriction and vasorelaxation of the arteries is easily determined in response to any agent or combination of agents of known concentration. Such isometric force recordings from isolated arteries have long been used to assess the function of arteries from animal models of diseases such as hypertension, atherosclerosis and diabetes and have thus revealed specific malfunctions in arteries associated with those conditions.

In addition, because many arterial rings can be prepared from a single artery from a single animal (up to 16 rings as 8 sets of duplicate samples in our laboratory), one experiment can be designed using the animal as its own control to examine effects of the presence or absence of any agonist, antagonist, toxin, stimulant, co-factor or new agent that may affect vascular reactivity. Such pharmacological manipulation of arterial function can also be used to reveal cellular mechanisms of an agent's action on arteries or to determine what cellular mechanisms are altered by a test agent. For example, arteries can be studied with and without their endothelium or with and without blockade of specific endothelial functions such as prostaglandin synthesis, production of endothelial autacoids or production of nitric oxide. This then can reveal whether a test agent acts through or modifies endothelial control of arterial contraction and whether that modification involves the important endothelial NO system.

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A Vascular Test Platform for the Evaluation of Potential Vasoactive and Vasoprotective Effects of Novel Botanical Compounds

Our laboratory has recently been engaged in extensive analysis of the vasoactive and vasoprotective properties of novel, anthocyanin enriched berry extracts derived from Chokeberry (*Aronia melanocarpa)*, Bilberry (*Vaccinium myrtillus)* and Elderberry (*Sambucus nigra)*. At the start of our studies in 2002 nothing was known about the vascular properties of these extracts. Therefore, we had to develop a series of independent studies, or a *Vascular Test Platform*, designed to answer four key questions that we believed should be addressed concerning the effects of a novel botanical agent on vascular function as they may relate to potential beneficial actions in cardiovascular disease. These questions are centered around evaluation of the potential vasoactive and vasoprotective properties of the compound and utilizes the *in vitro* isometric force recording technique described in the previous section. The primary questions used to evaluate vascular effects of novel botanical compounds are as follows,

- Is the botanical vasoactive?
- Are normal vascular responses altered in the presence of the botanical?
- Are normal vascular responses altered after sustained exposure to the botanical?
- Can the botanical protect arterial tissues from damage caused by exposure to reactive oxygen species?

Several additional questions, each addressing a particular point or testing for a relevant vascular mechanism, are contained within each primary question in the vascular test platform outlined above. A brief description of these additional questions within the four part vascular test platform is described below.

Test for Vasoreactivity of Novel Botanical Compounds

Detection of Vasoconstrictor and Vasodilator Properties

Vasoreactivity of blood vessels is the easiest vascular function to test experimentally and is best performed using isometric *in vitro* isometric force recording techniques in isolated arterial rings from experimental animals. In this technique test artery rings are contained within specially designed water jacketed glass receptacles (artery ring baths) that contain a pH and temperature controlled physiological salt solution of known volume [see reference (*12*) for details]. Any agent, such as a new botanical , can then be added at known concentrations to the baths containing the rings and any vasoreactivity responses of the rings recorded with any suitable recording device. In such recordings, vasoconstriction of arteries increases isometric force generated by the smooth muscle of the artery whereas vasorelaxation causes a reduction in any existing active contractile force on the artery. This method automatically reveals any potential direct effects of a novel agent on vascular reactivity.

With any compound undergoing vascular testing for the first time it will not be known whether the agent has vasodilator, vasoconstrictor, or both vasodilatory

In Flavor and Health Benefits of Small Fruits; Qian, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

and constrictor properties. With few exceptions arterial rings from any organ and animal do not exhibit any spontaneous intrinsic contraction (vascular tone) in *in vitro* preparations such as those used in artery baths. Thus, adding a novel agent to arteries in such *in* vitro settings where baseline contraction is absent can only reveal whether the agent has vasoconstrictive properties; there simply is no tone from which an agent could relax the artery. Consequently, it is better to pre-contract arterial rings in *in vitro* bath settings with an appropriate common physiological vasoconstrictor drug before applying any concentration of the test agent to the arteries. In this manner any vasodilator, as well as vasoconstrictor property, of the test agent can be revealed.

Certain procedures should be followed in experiments designed to measure vasoreactivity of artery rings *in vitro*. First, it is better to pre-contract all artery rings used in an experiment to the same approximate level of contraction before testing the response to a new compound rather than use the same concentration of vasoconstrictor drug in all rings used in the experiment. The reasons for this are two-fold. First, vasorelaxation of an artery ring is broadly inversely related to the level of preexisting contraction on that vessel. For example, it is relatively easy to relax an artery contracted to only 10% of its maximum possible contractile force, whereas it is difficult to relax arteries contracted near their maximum levels. Secondly, even artery rings taken from the same vessel in the same animal show variability in their contractile response to a single sub-maximal dose of a vasoconstrictor drug. Consequently, an investigator could inadvertently bias the interpretation of their experiments by using one dose of pre-contraction drug in all artery rings. Such treatment could inadvertently pre-contract one experimental group more than another. Resulting responses to a novel agent that happened to relax arteries, for example, would be automatically reduced in the higher pre-contracted group leading one to incorrectly assume that group was affected by the novel agent when in fact it was not. For this reason, all arterial rings used to test for potential vasoreactivity properties of a new agent should be titrated up to approximately the same percent of their individual maximum contraction before applying any dose of the agent, extract or preparation to be tested.

The selection of pre-contraction agent and agent used to determine the maximum contractile force in each artery ring depends on the type of artery used in given experiment. Arteries from organs other than the brain or heart can be maximally contracted with a 10 μM dose of norepinephrine which is an " --adrenergic vasoconstrictor. This natural neurotransmitter is the primary vasoconstrictor released onto arteries by vascular sympathetic nerves in mammals including humans. It is easy to titrate doses of norepinephrine in individual artery rings in order to create similar levels of pre-contraction in all rings prior to testing the vasoreactivity of a novel agent, such as a botanical. Unlike most arteries in the body, however, arteries from the brain and heart do not contract to norepinephrine and generally do not contract to any significant degree to many agents that are vasoconstrictive in other organs. For cerebral or coronay artery rings, a physiological salt solution containing a maximum cell depolarizing concentration of KCl (equimolar 80-130mM KCl replacing NaCl in the solution) can be used to determine maximal contractile force in those types of artery rings. However, because external KCl can antagonize the K^+ efflux vasodilator mechanism in

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arteries, it is generally not advisable to use KCl to produce pre-contraction on cerebral or coronary arteries in order to test potential vasodilation of a novel test agent on those vessels. Instead, U46619, a stable thromboxane analog can be used for that purpose because it can produce substantial contraction in arteries from the brain and heart. Regardless of the artery type used in a study, it is generally recommended that arteries be precontracted to ~30-40% of their individual maximum rather than 70% or more because the latter is generally considered a supra-physiological level of arterial contraction that can make it difficult to observe significant relaxation properties of a test agent.

With regards to the scope of vasoreactivity responses to examine with any new agent it is always better to examine arterial responses to several different concentrations of the novel agent rather than the reponse to a single dose. For this purpose the standard pharmacological procedure of creating a cumulative dose-response curve to the test agent should be performed. Once artery rings are precontracted to a similar percent of their maximum contractile force the new botanical agent to be tested can be added to the artery baths in cumulative half-log or full log increments from sub threshold to maximum response in the artery. (sub-threshold and maximum concentrations being determined in preliminary experiments designed for that purpose). In this manner a full vasoconstriction or vasorelaxation profile can be observed for the test agent. This allows the investigator to determine both the efficacy (potency) and sensitivity of the arteries to the test agent. The latter is generally indicated by determining an ED_{50} value for the new agent (the concentration of the agent that produces 50% of the maximum response to the agent) according to standard pharmacological principles.

Finally, if typical plasma levels of a test agent are known from other studies (for example, in studies that examine absorption and bioavailability of food components), a complete dose-response relationship will reveal whether the test agent is vasoactive at reasonable levels seen in the whole organism or whether responses represent only non-physiological, or supra-pharmacological vasoreactivity phenomena. Insight into whether a new botanical may be potentially beneficial in the context of cardiovascular disease depends on whether a realistic concentration of the new compound produces vasoconstriction or vasodilation in an artery and in what artery type those forms of vasoreactivity occur. Vasoconstriction in systemic arteries is a hypertensive stimuli that can also reduce blood flow to organs if it is too severe. Therefore, such vascular properties of a novel botanical would not be considered beneficial in cardiovascular disease. However, these agents could be beneficial in abnormal hypotensive settings such as circulatory shock if their vasoreactive properties did not include arteries from the brain or heart. Contraction of cerebral or coronary arteries by a novel botanical is always potentially dangerous and could be life threatening. This is true whether the test agent was hoped to be used for cardiovascular disease or whether it was intended for treatment of other diseases and conditions such as cancers, inflammation and infections. Generally speaking, vasodilation, which can reduce blood pressure and increase organ blood flow, can be considered a potential beneficial cardiovascular action of a new, previously untested agent. Furthermore, vasodilation of coronary or cerebral arteries can potentially mitigate

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ischemic conditions in those organs and theoretically reduce the probability of heart attack and stroke.

Determination of the Role of the Vascular Endothelium and NO in the Vasoactive Properties of Novel Botanical Agents

It is now generally advisable that the role of endothelium be investigated in any observed response of isolated arteries to a novel botanical agent because the vascular endothelium, especially through its NO system, plays such a major role in the vasoreactivity of blood vessels. It is useful for investigators of cardiovascular disease to know whether an new compound relaxes arteries, for example, through actions exclusive to the endothelium or whether the agent stimulates relaxation through a basic, direct action on arterial smooth muscle responsiveness to compounds released by the endothelium. For this reason, most vascular reactivity studies in the current literature use isolated artery rings with and without endothelium in their experimental design. Rings without endothelium are often prepared by gently rolling or scraping the inner surface of the artery with a forceps although more elaborate techniques may be used as well. Any vasoactive response of an arterial ring that was seen in rings with, but not without, their endothelium would indicate that the vasoactivity of the botanical test agent resided solely in mechanisms contained within the arterial endothelial cells and not with mechanisms associated with the arterial smooth muscle itself.

In addition, it is known that the vascular endothelium produces vasodilator prostaglandins such as prostacyclin as well as NO. Therefore, additional manipulation of artery rings and additional testing has to be done during an experiment to determine whether any new test agent works through the endothelium via the endothelial NO system. First, the impact of any basal prostacyclin production, or stimulated production by the botanical test agent, can be eliminated by treating arteries at the beginning of an experiment with a blocker of the arachidonic acid cascade that forms prostaglandins. Indomethacin or meclofenamic acid are good agents for that purpose (*13*). Next, during generation of the artery dose-response relationship to the novel botanical compound, the role of endothelial NO in any response to the botanical can be determined by adding an eNOS inhibitor (*13*) to the artery baths once the arterial response to the last dose of the botanical has reached its plateau. In this manner it can be determined whether the arterial endothelial NO system was stimulated by at least the maximum dose of the botanical compound being tested. For example, if the botanical compound relaxed artery rings with endothelium, but not in those without endothelium, and that relaxation could be abolished by addition of an eNOS inhibitor, then it can be concluded that the vasodilator properties of the novel botanical were due exclusively to its ability at activate the arterial endothelial NO system.

Evaluation of the NO system in the response of isolated arteries to novel botanical compounds has value to investigators of vascular function and cardiovascular disease beyond the straightforward information this yields about the direct vasoactivity of the botanical being tested. As discussed above, NO has many beneficial effects on arteries beyond its ability to cause vascular relaxation.

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Thus, in basic terms, proper vascular reactivity evaluations of novel botanical compounds can be used as surrogate markers of the ability of the compound to generally activate the endothelial NO system. Should a botanical demonstrate the ability to relax arteries by activating their endothelial NO system, then it is reasonable to assume that the botanical enhances NO production or bioavailability in arteries. This effect may impart a benefit in cardiovascular disease through non-vasoactive mechanisms such as inhibiting platelet aggregation and blood clot formation or attenuation of the pathogenesis of atherosclerosis.

Test for Alteration of Normal Vasoreactivity in Arteries Caused by the Presence of Novel Botanical Compounds

Even if a novel botanical agent is not intended for use in cardiovascular disease, such agents used for other purposes, once taken into the body, will unavoidably enter the plasma, extracellular fluid and possibly even cells throughout the body. Consequently blood vessels will not be able to avoid exposure to various concentrations of the botanical agent. For this reason it is important to examine whether the presence of a novel botanical agent can alter the normal functions of arteries and at what concentration of the botanical this may occur. It is very important to realize that an agent does not have to be directly vasoactive, nor does it need to exist at a vasoactive concentration in the body, in order to alter arterial reactivity. (The effect of sub threshold vasoactive concentrations of the neurotransmitter serotonin on contraction by other vasoconstrictors is but one example (*14*)). Thus whether responses of arteries to either naturally occurring or drug based vasoactive agents are altered by concurrent exposure to any novel botanical is an area of investigation that must be undertaken before potential biomedical benefits of that agent can be properly examined further. Also, a key issue in such evaluations is what type of vasoactive responses or vascular functions should be examined in the context of being potentially modified by the presence of a botanical agent. The choice of naturally occurring vasoactive agents, hormones and vascular neurotransmitters is very large and that of man-made pharmaceuticals with either vasoactive properties or the ability to affect other vascular functions is still large.

Considering the large impact of the endothelial NO system in modulating vascular reactivity and other important cardiovascular phenomena, evaluation of responses of arteries to endothelium dependent, NO-mediated vasodilators are a good first choice to examine whether a novel botanical compound can alter vascular function. Again, the *in vitro* isometric force recording technique describe above is ideally suited for such determinations. In such studies dose-response relationships of arteries with endothelium to a known endothelium dependent, NO-mediated vasodilator is tested in the presence or absence of a given concentration of the novel botanical agent. (*13*). In this manner it can be determined whether this botanical either enhances or impairs the efficacy or sensitivity of arteries to activation of the important endothelial NO system. There are several endothelium-dependent, NO-mediated vasodilators available that can be used to produce the relaxation dose-response curve. Acetylcholine is

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the prototypical receptor mediated agent of choice in most studies although it is a poor dilator in some arteries from some species (*15*). The calcium ionophore A23187 is a good, direct activator of eNOS and therefore produces endothelium dependent NO mediated vasodilation in arteries without the complexities of receptor activated processes in the artery, especially if it is used in arteries with prostaglandin synthesis blockade (*13*).

Although evaluation of potential effects of a new botanical on endothelium-dependent, NO-mediated vasorelaxation can yield important information to investigators of vascular function, such information alone is of limited value. Modification of endothelium-dependent, NO-mediated vasorelaxation by a botanical can occur at two levels in an artery; either the endothelial NO system itself is modified by the botanical or the endothelium dependent response is altered because the botanical has modified the sensitivity of the underlying arterial smooth muscle to NO. This distinction is important for fully understanding the impact of any novel agent on vascular function. Whether endothelium-dependent, NO-mediated vascular reactivity is altered by the presence of a botanical agent through effects at the endothelium or smooth muscle can be elucidated by adding assessment of the effect of the botanical on arterial relaxation to exogenous NO in the experimental design. Compounds that spontaneously release NO in aqueous media at physiological pH, such as DEA-NONOate (*13*) are ideal for this purpose. If responses to exogenous NO are unaffected by the presence of a botanical, then alteration of vascular sensitivity to NO is not involved in any observed alteration of responses to endothelium-dependent, NO-mediated vasodilators caused by the presence of the botanical. Instead these observations taken together would indicate that the botanical was acting exclusively at the level of the vascular endothelium.

Finally a choice needs to be made in such studies as to what dose of the novel botanical agent being tested should be used in acute exposure experiments. If a single component of a botanical extract is being examined and data is available about typical plasma levels seen for that component then that concentration is a logical first choice. However, such choices can not be made if the botanical being tested is a complex extract of many components. In that case it is best to choose a concentration of the extract that has been shown in other experiments to be non-vasoactive. Such a concentration simplifies interpretation of any results concerning alteration of vascular reactivity caused by the acute, concurrent presence of the botanical with the arteries.

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Test for Alteration of Normal Vasoreactivity in Arteries Caused by Sustained Exposure to Novel Botanical Compounds

As an extension to testing for whether normal vascular functions can be altered by acute concurrent exposure of arteries to novel botanical compounds, it is equally important to investigate whether longer term exposures to a new botanical can similarly alter vascular function. Again, as with acute testing experiments, the botanical need not be directly vasoactive nor present at vasoactive concentrations in order to alter vascular function. Also, it is not necessary for the botanical to exert acute alterations in vascular function in order to change vascular function following sustained exposure of vascular tissue to the botanical. This possibility is more likely if the novel test agent could work through alterations of gene induction within arterial tissue. Such genenomic effects typically require many hours to become fully expressed. A good example of this type of phenomenon is seen with the effects of a physiological concentration of beta-estradiol (1 nM) on endothelium-dependent, NO-mediated vasorelaxation of isolated rings of porcine coronary arteries (*16*). This dose of estradiol is not directly vasoactive on these arteries nor does it acutely alter endothelium-dependent, or independent NO-mediated vasorelaxation of those vessels. However, directly exposing arterial rings to 1nM beta-estradiol for 20-24 hours enhances the endothelial dependent NO mediated relaxation of the artery rings and enhances the ability of the arteries to withstand damage from reactive oxygen species (*17*). Both these effects are mediated through chronic actions of estradiol on the endothelium and not the arterial smooth muscle.

Classically, chronic exposure of arteries or any other tissue to a novel botanical is achieved by ingestion, infusion or sub-cutaneous implantation of the test substance into whole animal models or in human patients in controlled dietary studies. Although this method allows for exposure to the test agent for long periods of time it is difficult to determine whether the test agent alters vascular function through direct modification of an artery itself or whether such alteration results from changes in the physiology of the subject caused by chronic exposure to the test agent. A better experimental design would be to expose isolated arterial segments directly to known concentrations of the test botanical agent in controlled conditions that would preserve arterial viability over extended periods of time. However, exposure of arteries for many hours or days in artery baths used for *in vitro* analyses of vascular reactivity are not suitable for examining direct long term effects of a test agent on arteries because of problems associated with the non sterile nature of these baths. For that reason a sterile vascular tissue incubation technique was developed (*16*) that allows one to expose artery rings to any agent for up to 72 hours without loss of arterial viability and normal function. Arteries can then be removed from the incubation media and examined in classic *in vitro* isometric force recording systems without loss of normal vascular reactivity responses. This allows the investigator to determine whether alterations in vascular function occurred by direct sustained exposure of the arteries to the test agent.

Such sterile incubation techniques with isolated arterial rings could theoretically be used to test for long term direct effects of novel botanical compounds on various vascular functions. However a caution must be stated concerning interpretation of such studies. Ascribing potential beneficial vascular effects to a new botanical compound following exposure of arteries in typical tissue culture media for extended periods of time presumes that the novel compound does not deteriorate or is otherwise altered by the chemistry of the incubation media. This possibility points to the necessity of determining the stability of any new compound in any artificial tissue culture media before fully undertaking long term *in vitro* studies of the effects of botanicals on vascular tissue.

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Tests Used for the Determination of Whether Novel Botanical Agents Can Protect Arterial Function from Damage Caused by Exposure to Reactive Oxygen Species

It is well established that flavonoids, anthocyanins and other polyphenolic compounds exhibit strong oxygen radical scavenging capacity (*9*, *18*). This "anti-oxidant" property of these compounds, or the fruits and vegetables in which they are found , are often suggested as a potential biomedical benefit of these botanicals. However, the ability of a botanical to chemically quench oxygen radicals in bench top chemistry settings does not necessarily insure that the botanical will protect biological tissues from assault from reactive oxygen species. This is especially true if the concentration of the botanical exhibiting chemical anti-oxidant capacity far exceeds any reasonable concentration that could be reasonably expected to be seen in an individual following typical ingestion of the botanical in question.

Arteries are exposed to both external and internal oxygen radical assault in cardiovascular disease. Several investigators have examined the effects of reactive oxygen species on vascular function by exposing isolated arterial rings to either external or internally generated oxygen radicals in experimental settings such as *in vitro* isometric arterial force recordings. (*13*, *17*, *19*, *20*). Such procedures can be used to examine the damaging effects of oxygen radicals on vascular function and are ready made to test the potential anti-oxidant protective effects of novel agents (*19*, *20*). For example, *in vitro* vascular reactivity of isolated arteries (such as endothelium dependent and exogenous NO vasodilation as described above) can be examined following exposure of arteries to oxygen radicals in the presence or absence of a given concentration of a new botanical compound.

Testing for Protection of Arteries from External Exposure to Oxygen Radicals

Exposure of arteries to pyrogallol (*13*, *17*, *19*), potassium superoxide, the xanthine:xanthine oxidase reaction, or other agents can be used to cause damage to arteries from external reactive oxygen species (ROS) assault. However, to determine whether a new botanical protects or further impairs arterial function from suche oxygen radical assault, it is necessary that exposure of arteries to the ROS impairs, but does not totally destroy, the arterial function being tested. In so doing it is possible to observe whether the botanical being tested either improves arterial function (an anti-oxidant vasoprotective effect) or exacerbates impairment of such function (a detrimental oxidant effect) following exposure to ROS. A useful test, for example, would be to determine whether a botanical can protect against, or increase loss of endothelium NO mediated dilator capacity in arteries following their exposure to exogenous ROS.

As indicated in the pervious section, a proper concentration of the botanical for such anti-oxidant protection determinations needs to be selected for any experiment. Again, a concentration of the botanical that is not directly vasoactive in the artery type be examined is preferred over supra physiological concentrations, although higher concentration can be used if these are suggested to be reasonably and safely achieved in plasma of experimental animals or human subjects ingesting the botanical.

Testing for Protection of Arteries from Internal Exposure to Oxygen Radicals

Experimental tests of the efficacy of novel botanicals to protect arteries from external ROS assault have value for investigators of cardiovascular disease. However, such experiments essentially suggest that arteries are likely being protected by the radical quenching effect of the novel botanical agent in the solution surrounding the artery. A more demanding test of the ability of a botanical to protect arteries from ROS assault comes from experiments where isolated artery rings are exposed internally to excess ROS production. This can be accomplished by inactivating the superoxide dismutase (SOD) contained within the artery rings that normally inactivates O_2^- (*20*). In this manner, superoxide and resulting ROS build up inside the vessel. This causes arterial damage and loss of vascular responsiveness to agents such as endothelium dependent and independent NO vasodilators. For these experiments the duration of the internal radical exposure is selected to impair, but not totally abolish, the vascular reactivity response against which the botanical is being tested.

Internal ROS exposure of arteries provides the investigator with additional useful information as to the vasoprotective effect of a botanical agent. Were it to be demonstrated that such an agent protected an artery from, for example, a loss of NO mediated vasorelaxation following internal oxidant exposure, it would imply that the botanical was able to enter the cells of the artery or at least activate internal anti-oxidant mechanisms within the arterial cells. Such demonstrated ability of a novel botanical to provide anti-oxidant protection by being localized internally within the artery itself would be an especially beneficial effect of the botanical as a vascular protective agent in cardiovascular disease.

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Evaluation of the Vasoactive and Vasoprotective Effects of Three Anthocyanin Enriched Berry Extracts

We have recently used the vascular test platform described above to report on the vasoactive and vasoprotective properties of three novel anthocyanin enhanced extracts derived from Chokeberry (*Aronia melanocarpa)*, Bilberry (*Vaccinium myrtillus)* and Elderberry (*Sambucus nigra)* (*13*). These berries have some of the highest total anthocyanin contents and oxygen radical absorbance capacity (ORAC) reported for fruits and vegetables although their anthocyanin profiles in terms of type and percent composition differ (*13*). *In vitro* isometric force recordings of isolated coronary artery rings with and without endothelium from female pigs were used to examine dose-response relationships to each berry extract. The ability of a sub-vasoactive concentration of each extract to protect arterial reactivity from impairment following external exposure to ROS was also determined. For those studies the ability of each extract to prevent loss of vasorelaxation to the endothelium-dependent, NO mediated vasodilator A23187

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and exogenous NO from DEA NONOate was tested by examining dose-response relationships to each agent in the presence and absence of a berry extract with and without concurrent exposure to the superoxide donor pyrogallol. In addition, dose-response relationships of isolated coronary artery rings to A23187 and exogenous NO were determined in the acute presence of a sub-vasoactive concentration of each extract and following overnight sterile incubation of the rings in a tissue culture system to the same concentration of each extract.

The vasoactive profiles of each extract in isolated porcine coronary artery rings is shown in Figure 2. To examine potential vasoreactivity to the berry extracts all artery rings were precontracted to a similar percent of their individual maximum KCl contraction with U46619. The concentrations of each extract used were normalized to the same total anthocyanin concentration. Chokeberry and bilberry extract produced dose dependent vasorelaxation of porcine coronary arteries whereas such relaxation was not seen with elderberry extract. Furthermore relaxation was not seen to any concentration of any extract in rings that had their endothelium removed. In additional experiments it was shown that the relaxation of arteries to chokeberry and bilberry extracts could be totally abolished by application of the eNOS inhibitor, L-NO_2 arginine, to the arteries (data not shown).

Several conclusions can be drawn from these experiments. First, chokeberry and bilberry extracts are vasoactive in coronary arteries and their reactivity is exclusively vasodilatory. Futhermore, this vasorelaxation is consistent with the ability of these to extracts to stimulate the endothelial NO system of the artery. Results from these experiments further indicate that the endothelium dependent, NO mediated relaxation to these extracts can not be due to a simple non specific effect of the anthocyanins contained in the extracts. The total anthocyanin concentration used to construct the dose response relationships were the same for all extracts yet the elderberry extract did not relax artery rings. Furthermore, the relaxation responses seen could not be due exclusively to a similar non-specific effect of the total phenolic content of each extract because elderberry extracts reached total phenolic concentrations in the artery baths that were the same or greater than those in chokeberry and bilberry extracts that caused vasorelaxation. In terms of the concentration of specific components contrained in the chokeberry and bilberry extracts, vasorelaxation to those extracts start to occur at doses where concentrations of some individual anthocyanin are similar to plasma concentrations obtained in animal and human studies using oral ingestion of anthocyanin preparations. Taken in total these experiments suggest that individual variation in the vasorelaxation properties of these extracts may be due to specific components or combination of components in the chokeberry and bilberry extracts that are not present in extracts derived from the elderberries.

In separate experiments we observed that responses of isolated coronary artery rings to either A23187 or DEA NONOate were not affected by the presence of any berry extract at a sub-vasoactive concentration of 0.05 mg/L total anthocyanins nor were they affected by overnight incubation of artery rings in tissue culture media containing the same concentration of the berry extracts (data not shown).

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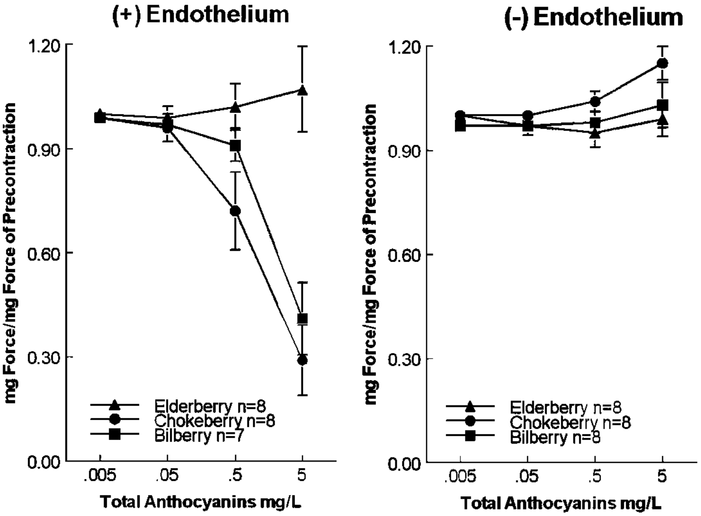

*Figure 2. Responses of isolated porcine coronary artery rings with (left) and without (right) endothelium to cumulative additions of anthocyanin enriched extracts from Elderberry, Bilberry and Chokeberry. Adapted with permission from J. Appl. Physiol. **2006**, 100, 1164−1170. Copyright 2005. The American Physiological Society.*

We have also reported that all three berry extracts at a sub-vasoactive concentration can protect coronary arteries from damage during exposure to external ROS (*13*). This is illustrated in Figure 3 which shows effects of a 0.05 mg/L total anthocyanin concentration of chokeberry extract on responses of coronary arteries to A23197 following exposure of the artery rings to superoxide released from the autooxidation of pyrogallol.

This figure shows that dose-dependent arterial relaxation, maximum relaxation and vascular sensitivity (ED_{50}) to A23187 is impaired in arteries exposed to pyrogallol but that this impairment is totally prevented by exposure of the arteries to chokeberry extract during the pyrogallol exposure. Similar results, although not totally preventitive, were seen when arteries were exposed to the same total anthocyanin concentration of either bilberry or elderberry extract. Furthermore we reported that no extract nor pyrogallol affected responses to DEA NONOate. Thus berry extracts at concentrations too low to directly alter coronary vascular tone protect coronary arteries from ROS exclusively through effects on the endothelial NO system and not through actions on arterial smooth muscle responsiveness to NO.

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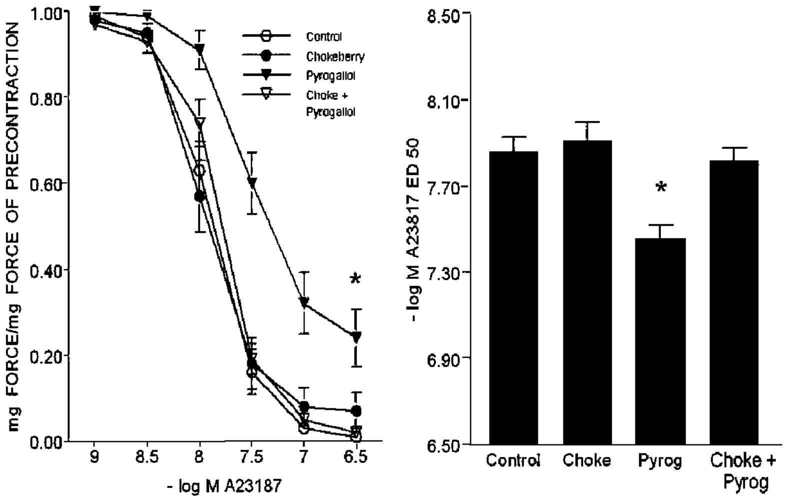

*Figure 3. Responses of isolated porcine coronary arteries to the endothelium dependant, NO-mediated vasodilator, A23187 following exposure to superoxide from pyrogallol with and without concurrent exposure to chokeberry extract at 0.05mg total anthocyanins/L. Dose dependent relaxation response to A23187 (left). Vascular sensitivity to A23187 (right) n=12 in all groups. * = p <0.05 versus control responses. Adapted with permission from J. Appl. Physiol.* ***2006****, 100, 1164−1170. Copyright 2005. The American Physiological Society.*

Current and Future Directions

The fact that anthocyanin enriched berry extracts have vasoactive and anti-oxidant vasoprotective effects on the coronary arterial endothelial NO system is significant. These specific properties could be especially beneficial in all forms of cardiovascular disease because such diseases are characterized by an impaired NO system and vascular damage due to exposure to excessive levels of reactive oxygen species.

The berry extracts listed in the previous section are complex entities containing many compounds of known and unknown quantities. We are currently engaged in several additional studies designed to further characterize the vasoactive and vasoprotective of these extracts in coronary arteries. Recent results indicate that these extracts can protect isolated coronary artery segments from damage arising from internal generation of ROS within the arteries. These effects appear to be mediated solely through the vascular endothelium and suggest that some anti-oxidant component of the extracts can enter vascular endothelial cells.

In preliminary experiments we have also observed individual anthocyanins contained within the berry extracts can produce vasorelaxation of coronary arteries. Some of these produce endothelium dependent NO mediated relaxation at very low concentrations. However, we have noted that a cocktail of anthocyanins

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of the type and concentration found in chokeberry extract does not relax arteries to the same extent as that seen in the complete extract. This indicates that components of the extracts other than anthocyanins may be vasoactive as well. Current studies in our laboratory have indicated that phenolic acids, especially those of the cinnamic acid family, are vasoactive in coronary arteries. These experimental results suggesting vasoactive properties of specific components of the berry extracts strongly suggest that these components should be examined for their potential to protect arteries from damage to either internal or external reactive oxygen species.

Finally, the pathogenesis of diseases such as atherosclerosis, hypertension and heart failure are far more complicated and multivariate than the single observation that they share an overexposure of the vasculature to oxygen radicals and a loss of NO mediated vascular functions. Although any agent that can attenuate oxygen radical mediated damage to an organism has theoretical value in the treatment of cardiovascular disease, this alone is not sufficient to claim broad health benefits from the consumption of that agent or the plant from which it is derived. Ultimately, the truest test of the potential cardiovascular benefit of any novel botanical agent must come in the form of the efficacy of the new agent in attenuating the pathological consequences of cardiovascular disease as reflected in improvement in the morbidity and mortality associated with such conditions.

References

1. Lloyd-Jones, D.; Adams, R.; Carnethon, M.; De Simone, G.; Ferguson, T. B.; Flegal, K.; Ford, E.; Furie, K.; Go, A.; Greenlund, K.; Haase, N.; Hailpern, S.; Ho, M.; Howard, V.; Kissela, B.; Kittner, S.; Lackland, D.; Lisabeth, L.; Marelli, A.; McDermott, M.; Meigs, J.; Mozaffarian, D.; Nichol, G.; O'Donnell, C.; Roger, V.; Rosamond, W.; Sacco, R.; Sorlie, P.; Stafford, R.; Steinberger, J.; Thom, T.; Wasserthiel-Smoller, S.; Wong, N.; Wylie-Rosett, J.; Hong, Y. Heart disease and stroke statistics—2009 update: A report from the American Heart Association statistics committee and stroke statistics subcommittee. *Circulation* **2009**, *119*, e21–e1812.
2. Vallence, P. In *Nitric Oxide Biology and Pathobiology*; Ignarro, L., Ed.; Academic Press: San Diego, CA, 2000.
3. Dillon, G. A.; Vito J. A. In *Nitric Oxide and the Cardiovascular System*; Loscalzo, J., Vito, J. A., Eds.; Humana Press: Totawa, NJ, 2000.
4. Harrison, D. G.; Ohara, Y. Physiologic consequences of increased vascular oxidant stresses in hypercholesterolemia and atherosclerosis: Implications for impaired vasomotion. *Am. J. Cardiol.* **1995**, *75*, 75B–81B.
5. Higashi, Y; Sasaki, S; Nakagawa, K; Matsuura, H; Oshima, T; Chayama, K. Endothelial function and oxidative stress in renovascular hypertension. *N. Engl. J. Med.* **2002**, *346*, 954–1962.
6. Langenstroer, P; Pieper, G. M. Regulation of spontaneous EDRF release in diabetic rat aorta by oxygen free radicals. *Am. J. Physiol.: Heart Cir.c Physiol.* **1992**, *32*, H257–H265.

7. Ma, X.; Weyrich, A. S.; Lefer, D.; Lefer, A. Diminished basal nitric oxide release after myocardial ischemia and reperfusion promotes neutrophil adherence to coronary endothelium. *Circ. Res.* **1993**, *72*, 403–412.
8. Galvano, F.; La Fauci, L.; Lazzarino, G.; Fogliano, V.; Ritieni, A.; Caippellano, S.; Battistini, N. C.; Tavazzi, B.; Galvano, G. Cyanindins: Metabolism and biological properties. *J. Nutr. Biochem.* **2004**, *15*, 2–11.
9. Wu, X.; Gu, L.; Prior, R. L.; McKay, S. Characterization of anthocyanins and proanthocyanins in some cultivars of Ribes, Aronia, and Sambucus and their antioxidant capacity. *J. Agric. Food Chem.* **2004**, *52*, 7846–7856.
10. Clarke, R.; Armitage, J. Antioxidant vitamins and risk of cardiovascular disease. Review of larger scale randomized trials. *Cardiovasc. Drugs Ther.* **2002**, *16*, 411–415.
11. Kromhout, J. Diet and the cardiovascular system. *J. Nutr. Health Aging* **2001**, *5*, 144–149.
12. Bell, D. R.; Bohr, D. F. Endothelium in functional aortic changes in coarctation hypertension. *Am. J. Physiol.: Heart Circ. Physiol.* **1991**, *26*, H1187–H1193.
13. Bell, D. R.; Gochenaur, K. Direct vasoactive and vasoprotective properties of anthocyanin-rich extracts. *J. Appl. Physiol.* **2006**, *100*, 1164–1170.
14. Bell, D. R.; Babcock, C. M.; Bohr, D. F. Individualities in post-serotonin attenuation and Na^+/K^+ pump activity in vascular smooth muscle. *Eur. J. Pharmacol.* **1989**, *171*, 189–199.
15. Cristie, M. I.; Griffith, T. M.; Lewis, M. J. A comparison of basal and agonist-stimulated release of endothelium-derived relaxing factor from different arteries. *Br. J. Pharmacol.* **1989**, *98*, 397–406.
16. Bell, D. R.; Rensberger, H. J.; Koritnik, D. R.; Koshy, A. Estrogen pretreatment directly potentiates endothelium-dependent vasorelaxation of porcine coronary arteries. *Am. J. Physiol.: Heart Circ. Physiol.* **1995**, *268*, H377–H383.
17. Bell, D. R.; Gochenaur, K. E.; Hecht, J. O_2^- mediated impairment of coronary arterial relaxation is prevented by overnight treatment with 1nM β-estradiol. *J. Appl. Physiol.* **2002**, *93*, 1952–1958.
18. Ehlenfeldt, M. K.; Prior, R. L. Oxygen radical absorbance capacity (ORAC) and phenolic and anthocyanin concentrations in fruit and leaf tissues of highbush blueberry. *J. Agric. Food Chem.* **2001**, *49*, 2222–2227.
19. Jackson, T. S.; Xu, A.; Vita, J. A.; Keaney, J. F. Ascorbate prevents the interaction of superoxide and nitric oxide only at very high physiological concentrations. *Circ. Re.s* **1998**, *83*, 916–922.
20. Lynch, S. M.; Frei, B.; Morrow, J. D.; Roberts, L. J.; Xu, A.; Jackson, T.; Reyna, R.; Klevay, L. M.; Vita, J. A.; Keaney, J. F. Vascular superoxide dismutase deficiency impairs endothelial vasodilator function through direct inactivation of nitric oxide and increased lipid peroxidation. *Arterioscler. Thromb. Biol* **1997**, *17*, 2975–2981.

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Chapter 10

Colonic Availability of Bilberry Anthocyanins in Humans

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Anthocyanins comprise a group of flavonoids and are important pigments in various plant species responsible for the distinctive colours of many flowers, fruits and vegetables. Dietary anthocyanins also receive considerable interest for their presumed roles in the prevention of various degenerative diseases. The aim of this study was to determine the amounts of anthocyanins reaching the colon after oral intake of bilberries (*Vaccinium myrtillus* L.), which were found to contain very high levels of anthocyanins (7834 mg/kg, compared to 365 mg/kg in cultivated blueberries according to quantitative analyses with authentic standards). After a polyphenol-free diet five healthy ileostomy volunteers each ate 300 g of bilberries. Ileostomy effluent was subsequently collected and anthocyanins in it were identified and quantified by HPLC-electrospray ionization tandem mass spectrometry and HPLC-diode array detection. In total, 46% ± 15% of the consumed anthocyanins were detected in the ileostomy effluent, with excretion peaking between one and four hours after intake. The results show that most of the bilberry anthocyanins were recovered in the ileostomy effluent, which would reach the colon under standard physiological

circumstances and contribute to the prevention of inflammatory bowel diseases and malignant gastrointestinal diseases.

Anthocyanins are a group of flavonoids capable of absorbing visible light and are therefore important pigments in various plant species (*1*). They are well known colouring agents in plants, being responsible for the distinctive colour of many flowers, fruits and vegetables (*2*). To date, 19 kinds of naturally occurring anthocyanidins - the aglycons of anthocyanins - have been reported (*2*) usually in conjugation with sugars at position 3-, 5-, 7-, 3'-, and 4'-. The most common anthocyanidins are pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin. The structure of the aglycon is a so called flavylium cation as shown in Figure 1. Anthocyanidins are usually glycosylated at the 3- or 5-position with monosaccharides such as glucose, galactose, rhamnose and arabinose, with disaccharides, or with trisaccharides.

Anthocyanidins are known to be unstable at pH above 2 (*3*); the flavylium cation is transferred into a chalcon structure via cleavage of the C-ring. Anthocyanins are stable either as glycosides, or in the so-called co-pigmented form with other flavonoids, but are fairly unstable in the deglycosylated form (*4*). Some anthocyanins are partially esterified with organic acids such as aromatic acids (caffeic, *p*-coumaric, ferulic, and sinapic acid) or aliphatic acids (acetic, malic, malonic, and oxalic acid) (*1*).

In the human diet the anthocyanin intake is estimated to be between 180 mg and 215 mg per day (*1*). Anthocyanins are present in vegetables such as cabbage, and especially in fruits like grapes, blueberries, cherries, red- and blackcurrants and strawberries (*5–7*). Between 2 g/kg and 4 g/kg of anthocyanins are reported in blackcurrant, blackberries, and blueberries or bilberries (*1*, *3*).

Blueberries, *Vaccinium spp.* (Ericaceae), are an important source of anthocyanins. As shown by some groups, several anthocyanins such as delphinidin-3-glycosides, malvidin-3-glycosides, cyanidin-3-glycosides, peonidin-3-glycosides, and petunidin-3-glycosides and their acetylated form (at position 6) are present in blueberries (*8*). However, the concentrations differ significantly depending on the blueberry variety (*9*). Bilberries (*Vaccinium myrtillus* L.), a wild type of blueberry, contain higher concentrations of anthocyanin than the cultivated (high-bush) blueberry variety *Vaccinium corymbosum* and also exhibit a correspondingly higher antioxidant capacity (*9*).

Today, dietary anthocyanins receive considerable interest for their presumed role in the prevention of various degenerative diseases. Intensively investigated physiological effects of these compounds are, in particular, their anti-oxidative properties (*10–13*). They are effective scavengers of reactive oxygen and radicals (*14*), and thus protect against cardiovascular diseases and cancer (*15*, *16*). Satué-Gracia has shown that anthocyanins can also protect against oxidation of low density lipoproteins (*17*).

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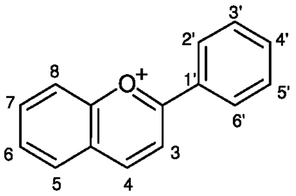

		position							
		3	5	6	7	3’	4’	5’	color at pH < 1
cyanidin	cy	OH	OH	H	OH	OH	OH	H	red
peonidin	pn	OH	OH	H	OH	OMe	OH	H	red
pelargonidin	pg	OH	OH	H	OH	H	OH	H	orange
malvidin	mv	OH	OH	H	OH	OMe	OH	OMe	violet
delphinidin	dp	OH	OH	H	OH	OH	OH	OH	violet
petunidin	pt	OH	OH	H	OH	OMe	OH	OH	violet

Figure 1. Structures and colours of the anthocyanin aglycons (called anthocyanidins) investigated at pH < 2 (2-phenyl-1-benzopyrilium, flavylium cation).

The protective effects of anthocyanins in the prevention of inflammatory diseases and colon carcinogenesis, have been investigated in several research projects. In studies with cell cultures and animal models, anthocyanins have shown positive effects on oxidative stress responsive proteins such as nuclear factor-kappa B ($NF_{\kappa}B$) (*18*, *19*), and on anti-proliferative activities in colon cancer cell lines (*20*) and intestinal carcinogenesis (*21–23*). A juice rich in anthocyanins has been shown to decrease the markers of oxidative stress (*24*). Recently an anthocyanin-rich berry juice has shown protective effects on oxidative stress in hemodialysis patients (*25*).

Blueberries are well known in traditional folk medicine for the treatment of degenerative and inflammatory diseases of the colon (*26*). Using an animal model, some groups have shown that anthocyanins do reach the colon after the ingestion of anthocyanin rich food (*27*, *28*). However, data concerning the amount of anthocyanins reaching the colon after the consumption of bilberries are limited. In order to quantify this process in our study, human subjects with a terminal ileostomy consumed 300 g of bilberries, and the anthocyanin content was measured in ileostomy effluents. Blueberries and bilberries were also analyzed for their anthocyanin contents in order to compare the concentrations available in wild and cultivated forms, and to know the exact amount administered to the experimental subjects and hence estimate the passage of unmetabolized anthocyanins to the colon.

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Materials and Methods

Chemicals

All chemicals and solvents were of analytical grade and obtained from the following sources: Acetonitrile (Lichrosolv®) from Merck (Darmstadt, Germany); formic acid from Fluka (Deisenhofen, Germany); Methanol (HPLC grade) and hydrochloric acid from Fisher Scientific (Louhgborough, UK); internal standard 3,4,5-trihydroxycinnamic acid from Aldrich (Steinheim, Germany). Solvents were redistilled before use.

Blueberries

Cultivated (highbush) blueberries (*V. corymbosum*), and commercially available (lowbush) bilberries (*V. myrtillus*) (country of origin: Germany) were purchased from a local market. The fruits were ground separately and a 5 g portion was drawn from each sample for subsequent quantification of anthocyanin content. Five 300 g portions of bilberries were used in the dietary intervention study.

Isolation of Anthocyanins

Bilberries and blueberries were extracted for anthocyanin quantification with 500 mmol HCl in methanol. For this, 5 g were homogenized with an ULTRA TURAX® and extracted twelve times. The anthocyanins were quantified with HPLC-diode array detection (HPLC-DAD) analysis.

Authentic anthocyanins were sourced from a 300 g sample of low-bush bilberries (*V. myrtillus*) which were homogenized and extracted three times with 300 ml of acetonitrile/water/formic acid 49/50/1 (*13*). After evaporating and lyophilizing, 19 g of raw extract was dissolved in aqueous 2% formic acid, and transferred onto a glass column filled with Amberlite XAD-7 (35 x 4 cm), pre conditioned with 2% aqueous formic acid, and was washed with 1 l water containing 2% formic acid to remove sugars, organic acids, proteins and salts. Phenolic compounds, such as anthocyanins, were retained by the resin and eluted using 1.5 l from a mixture of acetonitrile/water/formic acid 49/49/2. The XAD-7 resin was cleaned by washing with acetonitrile/formic acid 98/2. Acetonitrile was evaporated *in vacuo* and the aqueous solution was lyophilized using a Christ Alpha 1-4 apparatus (Osterode, Germany).

For the isolation of authentic anthocyanins, the polyphenol fraction was dissolved in aqueous 10% formic acid and injected in a semi-preparative HPLC system, which consists of two Knauer-64 pumps, a Knauer mixture chamber and a Knauer UV/VIS-detector (Knauer, Berlin, Germany). A Eurosphere100 column 250 x 4.6 mm, with 5 µm particle size (Knauer, Berlin, Germany) was used. The mobile phase consisted of aqueous 10% formic acid (A) and acetonitrile (B) (v/v). The gradient applied was 2-5% B from 0-1 min, 5% B from 1-3 min, 5-6%

B from 3-6 min, 6 % B from 6-8 min, 6-10% B from 8-30 min, 10-20% B from 30-45 min, 20-99 % B from 45-46 min and 99% B from 46-50 min; a flow rate of 1.1 ml/min was used and 1 mg anthocyanins dissolved in aqueous 10% formic acid was injected (injection volume 50 µl, 150 times). Only two anthocyanins could not be separated on the column used. To separate these, an Agilent Zorbax™ SB-C8 column, 150 x 4.6 mm, with 5 µm particle size (Agilent Technologies, Waldbronn, Germany) was used with the same gradient as described above. The isolated anthocyanins were characterized by ^{1}H-NMR, ESI$_{pos}$-LC-MS/MS and UV/VIS-spectra.

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HPLC-DAD Analysis

The HPLC system used was a Hewlett-Packard 1100 HPLC gradient pump and a Hewlett-Packard 1100 photodiode array detector (Waldbronn, Germany), equipped with a Wisp 710b autosampler (Waters, Eschborn, Germany). Data acquisition and evaluation were performed with a Hewlett-Packard Chemstation and associated software. A Zorbax™ SB-C8 column, 150 x 2.1 mm, with 3.5 µm particle size, (Agilent Technologies, Waldbronn, Germany) was used. The mobile phase consisted of aqueous 10% formic acid (A) and acetonitrile (B) (v/v). The gradient applied was 2% B from 0-3 min, 2-5% B from 3-17 min, 5-6% B from 17-28 min, 6-15% B from 28-35 min, 15-30% B from 35-45 min, 30-99% B from 45-50 min, and 99% B from 50-55 min; a flow rate of 0.35 ml/min and 20 µl injection volumes were used. The peaks were identified by comparison of retention time and UV/VIS spectra (200 to 600 nm) with authentic references. Anthocyanins were determined at 520 nm and the internal standard 3,4,5-trimethoxycinnamic acid at 280 nm.

HPLC-DAD-MS/MS Analysis

HPLC-electrospray ionization tandem mass spectrometry (HPLC-DAD-ESI-MS/MS) was performed with a TSQ 7000 tandem mass spectrometer system equipped with an ESI interface (Finnigan MAT, Bremen, Germany), and a Hewlett-Packard 1100 HPLC gradient pump and a Hewlett-Packard 1100 photodiode array detector (Waldbronn, Germany), equipped with a Wisp 710b autosampler (Waters, Eschborn, Germany). MS-data acquisition and evaluation were conducted on a DEC 5000/33 (Digital Equipment, Unterföhring, Germany) using Finnigan MAT ICIS 8.1 software. Data acquisition and evaluation were performed with a Hewlett-Packard Chemstation and associated software. HPLC chromatographic separations were carried out on a Zorbax SB-C8 column, 150 x 2.1 mm, with 3.5 µm particle size (Agilent Technologies, Waldbronn, Germany). The mobile phase consisted of aqueous 10% formic acid (A) and acetonitrile (B) (v/v). The gradient applied was 2% B from 0-3 min, 2-5% B from 3-17 min, 5-6% B from 17-28 min, 6-15% B from 28-35 min, 15-30% B from 35-45 min, 30-99% B from 45-50 min, and 99% B from 50-55 min; a flow rate of 0.35 ml/min and 20 µl injection volumes were used. The analysis was performed in the positive ionization mode. The spray capillary voltage was set to 3.2 kV, and the temperature of the heated capillary was 200 °C. Nitrogen served both as sheath (70 psi) and auxiliary gas (10 units). The mass spectrometer was operated in the full-scan mode, *m/z* 150 - 1000, with a total scan duration of 1.0 s. MS/MS experiments were performed at a collision energy of 20 eV, with argon (2.0 mTorr) serving as collision gas. The molecular ion peaks and mass spectra obtained were compared to those of previously measured references.

NMR Analysis

The isolated authentic references were determined by one-dimensional ^{1}H-NMR. NMR spectra were recorded on a Bruker AVANCE 400 NMR spectrometer (Rheinstetten, Germany). For ^{1}H-NMR measurement, anthocyanins were dissolved in D_2O containing 2% DCl. NMR data are not presented here.

Subjects

The study protocol was approved by the Ethics Committee of the Medical Faculty, University of Wuerzburg, Germany. After giving their informed written consent, five otherwise healthy female subjects (mean age 35 years, range 23 to 47 years), each with an end ileostomy, participated in the trial. They had undergone colectomy with terminal ileostomy prior to the study; the underlying conditions were Crohn´s disease (n = 4) or familial adenomatous polyposis (n = 1). Patients who had been resected for colonic Crohn's disease had no evidence of small intestinal involvement. Concerning the type of operation, only those patients in whom no ileal resections had been performed were recruited into the study.

Study Design

Volunteers avoided food containing polyphenols the day before the study. After a 10 hour overnight fast they consumed, within 15 minutes, a bilberry meal in the form of 300 g puréed bilberries (*V. myrtillus*) with 100 g skimmed-milk yoghurt (0.1% fat). The subjects fasted for a further four hours and were then served a polyphenol-free, light meal. The ileostomy bags were collected immediately before and 0.5, 1, 2, 4, 5, 6 and 8 hours after starting to ingest the bilberry purée. The contents of the ileostomy bags were immediately frozen at -24 °C and stored before extraction and analysis.

Preparation of ileostomy fluids. The frozen ileostomy fluids were freeze-dried using a Christ Alpha 1-4 apparatus (Osterode, Germany) and homogenized. Aliquots (0.2 g) were extracted ten times with 2.0 ml methanol containing 500 mmol HCl, sonicated with an ultra-sonic probe (Elma Transsonic 460, Singen, Germany) for 10 min and centrifuged at 6.000 rpm for 6 min (Hettrich EBA-12, Tuttlingen, Germany). The solvent was evaporated at 35 °C, and the extract dissolved in 5 ml aqueous 10% formic acid containing 400 µl methanol. To

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determine recovery rates of sample preparations, references were added to a polyphenol-free ileostomy fluid. Sample preparations were performed as described above. Recovery rates ranged between 85% and 99% (n = 3).

Quantification of Blueberries/Bilberries and Ileostomy Fluids

Aliquots from stock solutions (between 42.2 mg/l and 79.8 mg/l) of the authentic anthocyanins isolated from bilberries in aqueous 10% formic acid, were diluted (range from 0.7 mg/l to 40 mg/l) and 3,4,5-trihydroxycinnamic acid (50 mg/l) was added (v/v = 1/1) as an internal standard.

Calibration curves (at the appropriate wavelengths according to the absorption maxima of the compounds) were used for quantification. Anthocyanins were quantified by means of calibration curves (peak area divided by internal standard area, versus quotient of polyphenol and the internal standard concentration). Linearity was given for 0.7 mg/l to 40 mg/l; limit of quantification was 0.35 mg/l; limits of determination were 0.2 mg/l with a signal to noise ratio of 3:1 (*29*) respectively. All experiments were performed in triplicate. Compounds were identified by comparison of retention time, UV-spectra and MS as well as MS/MS information using reference compounds as previously demonstrated.

No standard was given for peonidin-3-*O*-galactoside. Instead, this compound was identified using HPLC-ESI-MS/MS and quantified as peonidin-3-*O*-glucoside by HPLC-DAD.

Prior to HPLC-DAD and HPLC-MS/MS analyses, blueberry and bilberry extracts, and ileostomy fluid extracts were centrifuged, the internal standard was added, and the samples were analyzed. The identification and quantification of anthocyanins in blueberries, bilberries, and ileostomy effluents were performed using HPLC-DAD and HPLC-ESI-MS/MS in the negative mode as described previously (*8*, *13*, *30*, *31*). Structural elucidation was performed by comparing the spectroscopic data with those of the isolated references.

Enzymatic Activity and Microbiological Spectra

Fresh, polyphenol-free ileostomy fluids (t=0) were used immediately for ApiZYM Assays (Biomérieux, Marcy l'Etoile, France). Microbiological determinations of aerobic and anaerobic microorganisms in fresh ileostomy effluents were performed at a microbiological laboratory (L+S AG, Bad Bocklet, Germany).

Results

In our study fourteen anthocyanins were isolated from bilberry using semi-preparative HPLC. These included delphinidin-3-*O*-galactoside (dp-3-gal), delphinidin-3-*O*-glucoside (dp-3-glc), delphinidin-3-*O*-arabinoside (dp-3-ara),

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cyanidin-3-*O*-galactoside (cy-3-gal), cyanidin-3-*O*-glucoside (cy-3-glc), cyanidin-3-*O*-arabinoside (cy-3-ara), petunidin-3-*O*-galactoside (pt-3-gal), petunidin-3-*O*-glucoside (pt-3-glc), petunidin-3-*O*-arabinoside (pt-3-ara), peonidin-3-*O*-galactoside (pn-3-gal), peonidin-3-*O*-glucoside (pn-3-glc), malvidin-3-*O*-galactoside (mv-3-gal), malvidin-3-*O*-glucoside (mv-3-glc), and malvidin-3-*O*-arabinoside (mv-3-ara), which were identified using HPLC-DAD, ^{1}H-NMR and HPLC-ESI-MS/MS for structural elucidation. All spectroscopic data were in good agreement with other data previously reported in the literature (*8*, *13*, *30*, *32*). These standards were used to determine the anthocyanin contents of commercially available (low-bush) bilberries (*V. myrtillus*) and cultivated (high-bush) blueberries (*V. corymbosum*). The results are shown in Table 1.

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Table 1. Anthocyanin content (mg/kg) of bilberries used in the ileostomy study vs. cultivated highbush blueberries, as determined by HPLC-diode array detection at 520 nm using authentic standards[1]

Compound	*Bilberries*	*Blueberries*
Delphinidin-3-*O*-galactoside	1222 ± 64	29.4 ± 1.7
Delphinidin-3-*O*-glucoside	1663 ± 151	38.8 ± 3.4
Delphinidin-3-*O*-arabinoside	695 ± 13	17.4 ± 0.6
Cyanidin-3-*O*-galactoside	255 ± 22	7.2 ± 0.1
Cyanidin-3-*O*-glucoside	414 ± 32	9.3 ± 0.4
Cyanidin-3-*O*-arabinoside	291 ± 20	4.3 ± 0.5
Petunidin-3-*O*-galactoside	536 ± 36	34.7 ± 1.4
Petunidin-3-*O*-glucoside	1119 ± 83	60.6 ± 1.2
Petunidin-3-*O*-arabinoside	326 ± 22	18.2 ± 2.7
Malvidin-3-*O*-galactoside	384 ± 63	65.7 ± 5.2
Malvidin-3-*O*-glucoside	354 ± 29	39.4 ± 1.0
Malvidin-3-*O*-arabinoside	189 ± 27	25.9 ± 5.0
Peonidin-3-*O*-galactoside	24 ± 2	0.7 ± 0.2
Peonidin-3-*O*-glucoside	363 ± 13	13.5 ± 4.1
Sum of anthocyanins	7834	365

[1] mean ± SD; n = 2.

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For bilberries, the total anthocyanin concentration was 7834 mg/kg, whereas for cultivated blueberries it was only 365 mg/kg. In bilberries, the highest individual anthocyanin concentration was determined as the dp-3-glc with 1663 mg/kg, whereas in cultivated blueberries the highest individual anthocyanin concentration was of mv-3-gal at 66 mg/kg. Generally, dp-3-glc, dp-3-gal (1222 mg/kg), and pt-3-glc (1119 mg/kg) dominated the anthocyanin spectra of bilberries, whereas in high-bush blueberries in addition to mv-3-gal, pt-3-glc at 61 mg/kg was the only other main anthocyanin present. No free anthocyanidin aglycons or acylated anthocyanins were detectable in either of the *Vaccinium* species under study using HPLC-DAD at 520 nm.

Anthocyanin concentrations in the ileostomy fluids of the five ileostomy patients after they each consumed 300 g of bilberries were determined using HPLC-DAD analysis at 520 nm directly after the sample preparation procedure. Stability of anthocyanins throughout the sample preparation procedure was proven by adding references to a polyphenol-free ileostomy fluid: no anthocyanin degradation was observed.

Structural elucidation of all compounds under study was performed by means of retention time, UV maximum and HPLC-ESI-MS/MS fragmentation pattern as shown previously. A HPLC-DAD-chromatogram (λ = 520 nm) of an ileostomy extract, taken two hours after the patient had consumed 300 g of bilberry purée, is shown in Figure 2. Dp-3-gal, dp-3-glc, cy-3-gal, dp-3-ara, cy-3-glc, pt-3-gal, cy-3-ara, pt-3-glc, pn-3-gal, pt-3-ara, pn-3-glc, mv-3-gal, mv-3-glc, mv-3-ara, and 3,4,5-trimethoxycinnamic acid as internal standard (IS, λ_{max} = 280 nm) were identified.

Time courses of the total blueberry anthocyanins occuring in the ileostomy fluids of all five volunteers during the eight hour period following consumption is given in Figure 3, and for each single proband in Figure 4.

The highest concentrations of anthocyanins appeared in the ileostomy bag one hour after ingestion. Two of the five participants showed two excretion maxima of anthocyanins in the ileostomy effluent: the first at one hour and the second at four hours after ingestion. Data of the five volunteers are given showing their individual variation in absorption or metabolization. In total, 46% ± 15% of the consumed anthocyanin was detected in the ileostomy effluent.

The detailed occurrence of single compounds in the ileostomy fluids, over the eight hour time course following ingestion is shown in Figures 5 and 6.

For dp-3-gal, dp-3-glc, dp-3-ara, cy-3-glc, pt-3- glc, pt-3-gal, and mv-3-glc maximum excretion was observed one hour after consumption as shown in Figure 5. Similarly, for the anthocyanins cy-3-gal, cy-3-ara, mv-3-gal, mv-3-ara, pn-3-glc, and pt-3-ara maximum excretion was observed in the ileostomy bag between one and four hours after consumption (Figure 6). All anthocyanins as determined in the ileostomy fluids, had completely passed the small intestine six hours after bilberry consumption. No anthocyanidin aglycons were detectable in the ileostomy effluents.

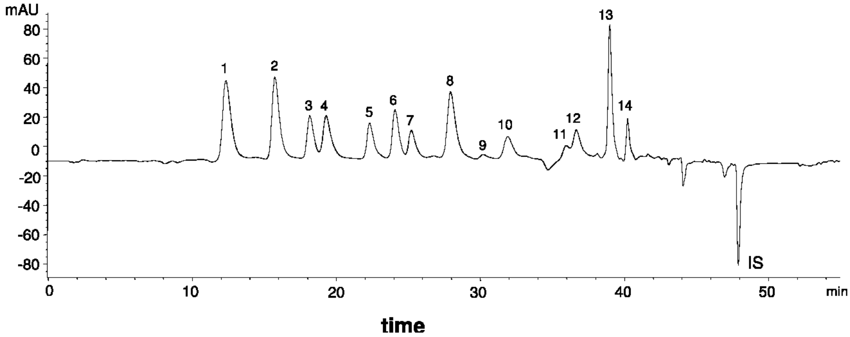

Figure 2. HPLC elution profile of ileostomy extract at 520 nm 2 hours after bilberry consumption. Unlabelled peaks are intestinal constituents. IS = internal standard (3,4,5-trimethoxycinnamic acid). Delphinidin-3-O-galactoside (1), delphinidin-3-O-glucoside (2), cyanidin-3-O-galactoside (3), delphinidin-3-O-arabinoside (4), cyanidin-3-O-glucoside (5), petunidin-3-O-galactoside (6), cyanidin-3-O-arabinoside (7), petunidin-3-O-glucoside (8), peonidin-3-O-galactoside (9), petunidin-3-O-arabinoside (10), peonidin-3-O-glucoside (11), malvidin-3-O-galactoside (12), malvidin-3-O-glucoside (13), and malvidin-3-O-arabinoside (14).

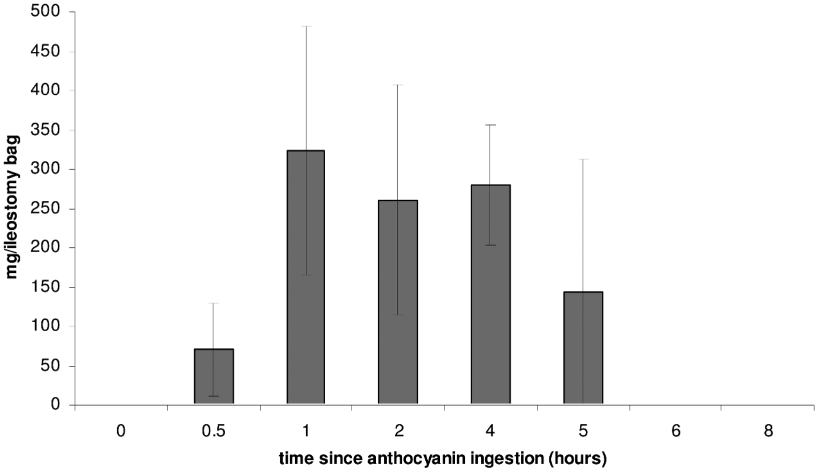

Figure 3. Time course of total anthocyanin excretions (mg/ileostomy bag) in the ileostomy fluids of patients (n=5) under study. Data are expressed as mean ± SD.

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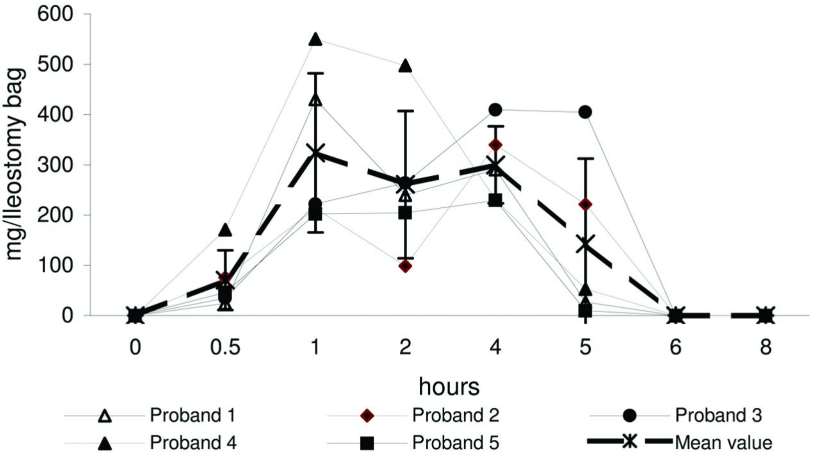

Figure 4. Time course of excretion of total anthocyanins (mg/ileostomy bag) in the ileostomy fluids of single patients and mean values ± SD are shown.

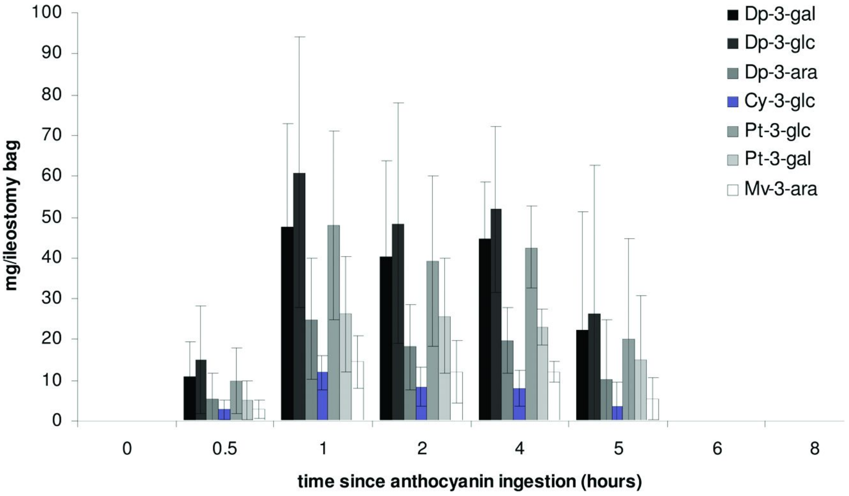

Figure 5. Time dependent excretion of single anthocyanins (dp-3-gal, dp-3-glc, dp-3-ara, cy-3-glc, pt-3-glc, pt-3-gal, and mv-3-glc) (mg/ileostomy bag) in the ileostomy fluids of patients (n = 5) under study. Data are expressed as mean ± SD.

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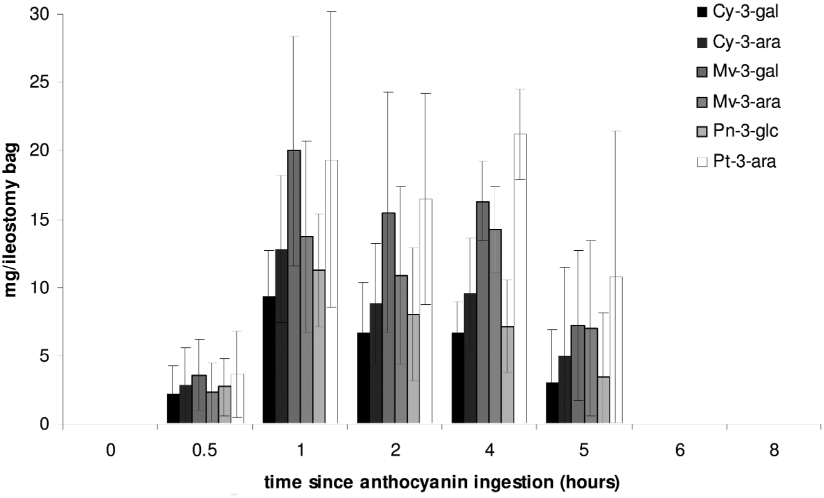

Figure 6. Time dependent excretion of single anthocyanins (cy-3-gal, cy-3-ara, mv-3-gal, mv-3-ara, pn-3-glc, and pt-3-ara) (mg/ileostomy bag) in the ileostomy fluids of patients (n = 5) under study. Data are expressed as mean ± SD.

High β-glucosidase, α-glucosidase, and β-galactosidase activity as well as esterase and phosphatase activity were observed in ileostomy effluents. The investigations of anaerobic microflora in the ileostomy bags demonstrated the total germ numbers of three samples of fluid, as determined by standard microbial diagnostics, to be *Bacteroides sp.*: less than 10^8/g and *Bifidobacterium sp.*: between 10^7/g and 10^9/g. Large differences between individuals were observed.

Discussion

In our study, the total anthocyanin concentration in bilberries was 7834 mg/kg, dominated by dp-3-glc with a concentration of 1663 mg/kg; whereas for cultivated blueberries the total anthocyanin concentration was only 365 mg/kg. Our data agree well with those of Clifford who reported total anthocyanin concentrations ranging from 0.82 g/kg to 4.2 g/kg (*1*) and Mazza and Miniati (*3*) or Sellapan et al. (*33*) reporting 0.25 g/kg to 5.0 g/kg or 841 mg/kg fresh weight. In the literature, the highest anthocyanin concentrations were also determined for bilberries with an average of 5997 mg/kg dominated by dp-3-gal, dp-3-glu, and dp-3-ara (*13*) whereas the main blueberry anthocyanins were reported by Prior and co-workers to be mv-3-glc, mv-3-ara, and pt-3-glc (*8*). Total anthocyanin concentrations in our study of bilberries were slightly higher than those reported elsewhere in the literature. This is probably due to the fact that, for the first time, authentic standards were used whereas previous studies have calculated cy-3-glc equivalents (*13*). In general, bilberries provide much higher concentrations of anthocyanins than blueberries.

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Information on the bioavailability of anthocyanins in humans is limited to a few studies that have investigated plasma levels and urinary excretion after consumption of anthocyanin rich food (*1*). For example, Fleschhut *et al.* investigated the bioavailability of cy-3-glc in humans by measuring plasma and urinary levels of the glycoside (*34*). In that study, cy-3-glc, cy-3-glucuronide, the cyanidin aglycon, and the methylated product pn-3-glc, as well as peonidin-3-glucuronide were all found in plasma and urine. After the consumption of blueberry extracts 0.004% of the consumed dose was recovered as unchanged glycosides, methylated metabolites, and mono-glucuronides in the urine of women (*35*). Felgines *et al.* identified glucuronides, sulphates, and the unconjugated aglycon after the consumption of strawberry pelargonidin glucoside (*36*) and Mazza *et al.* detected 19 of 25 anthocyanins in human plasma after the consumption of blueberry anthocyanins (*37*) where levels of absorption were 0.002% to 0.003% of the ingested dose. Some studies investigating urinary excretion levels after consumption of red grape juice and red wine anthocyanins have demonstrated 0.23% and 0.18% respectively to be recovered unchanged in urine within seven hours of consumption (*38*). In a study of *Hibiscus sabdariffa* anthocyanins, Frank *et al.* measured 0.018% of the consumed intake in urine. The maximum plasma levels occurred 1.5 hours after anthocyanin consumption whereas in urine, maximum concentrations were observed after 1.5 to two hours (*39*). This agrees with the data of McGhie *et al.* who reported urinary excreted anthocyanins to be at a maximum two hours after blueberry consumption, with less than 0.1% of the administered dose being observed (*40*). Other groups have also reported unmetabolized anthocyanins in human urine samples (*41*, *42*) and in general, most pharmacokinetic studies have shown very poor bioavailability of all compounds under study after the consumption of anthocyanin rich foods or pure anthocyanins.

Inflammatory bowel diseases (IBD) and colorectal cancer are two of the main types of degenerative illness in Western countries. It is known that anthocyanin rich foods show preventive effects in oral (*43*), esophageal (*23*) and colorectal (*27*, *44*) malign degenerations. Determining the compounds active in the prevention of such diseases is, therefore, of vital importance. Model studies have shown that anthocyanins are rapidly deglycosylated in the colon into low molecular weight compounds (*45–47*) which also occur in the urine of probands (*48*). How many anthocyanins, and how much, reaches the colon remain questions of great importance. To date, no data have been available concerning the colonic availability of anthocyanins in humans. Only data on the colonic availability of single compounds, such as quercetin chlorogenic acid (*49–52*) and cloudy apple juice (*31*, *53*), have been studied using ileostomy patients. Recently, He *et al.* and Borges published data on the amounts of anthocyanins in the colon and faeces of rats (*27*, *28*).

Our results show that after the ingestion of bilberries, between 28.3% and 85.1% of the consumed anthocyanins reach the end of the small intestine unmetabolized (*31*). The anthocyanin spectrum of bilberries appeared almost unchanged in the ileostomy effluent, being dominated by dp-3-glc, dp-3-gal, and pt-3-glc, whereas differences in the absorption or degradation rates of anthocyanin glucosides, galactosides, and arabinosides were obvious. Glucosides were absorbed or metabolized more intensively than galactosides. The arabinosides were the least absorbed or metabolized since these, with the exception of delphinidin arabinoside, were recovered in the highest concentrations from the ileostomy bags. Delphinidin arabinosides were, however, absorbed more intensively, with 37.8% of the consumed amount being recovered in the ileostomy bags, than the corresponding glucosides and galactosides which were recovered at rates of 40.7% and 45.3% respectively. These findings are in good agreement with our recently published data which showed that while quercetin-3-*O*-rhamnoside and quercetin-3-*O*-arabinoside occurred in the ileostomy fluids, all other quercetin glycosides such as quercetin 3-*O*-glucoside were not detectable (*31*, *53*). These findings are also in good agreement with the *ex vivo* fermentations of quercetin and artificial *p*-nitrophenol glycosides by Knaup *et al.* (*54*) which showed a relationship between the sugar moiety and intestinal degradation. Also in accord with our findings are those of Mc Ghie *et al.* and Wu *et al.* who have reported differences in the urinary excretion rate of blueberry anthocyanins to be dependent on the sugar moiety and the nature of aglycon respectively (*35*, *40*). As mentioned above, He *et al.* have reported different stabilities of anthocyanin glucosides, galactosides and arabinosides in the gastrointestinal trace of rats: glucosides degrading rapidly in the small intestine, whereas arabinosides remain stable (*27*). They have concluded, therefore, that anthocyanin pentosides reaching the colon could play an important role in the prevention of colorectal cancer. A correlation between faecal anthocyanin content and a lower incidence of aberant crypt foci (ACF) at an early stage of colon cancer in rats, has been reported in that study and others (*22*, *23*).

In agreement with data acquired in a study of ileostomists who consumed cloudy apple juice, high variations of the excretion rates were observed among individuals (*55*).

Considering the recovery rates of malvidin, petunidin, and cyanidin glycosides in the ileostomy fluids, their metabolism seems to be dependent on the sugar moiety bound to the anthocyanidin aglycon. Glucosides are degraded or absorbed to a higher extent than galactosides, whereas of the three, the arabinosides are recovered in the highest concentration from the ileostomy fluids (*31*). Free anthocyanin aglycons did not occur in the ileostomy fluid. Only the recovery pattern of delphinidin glycosides from the ileostomy bags was different. In contrast to malvidin, petunidin, and cyanidin glucosides, the del-3-glc was not absorbed or metabolized to a high degree. Considering the nature of aglycon, malvidin and petunidin glycosides were absorbed or metabolized less than cyanidin and delphinidin conjugated anthocyanins. Here, malvidin and petunidin possess methoxy groups whereas cyanidin and delphinidin both have a hydroxyl group at the B-ring (Figure 7).

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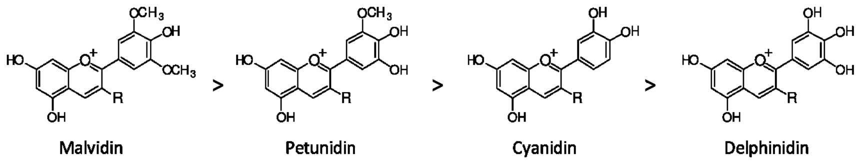

Figure 7. Colonically available anthocyanins. Methoxylated anthocyanins are more available in the colon than the hydroxylated compounds under study (R = glycosides).

The metabolization of anthocyanins, and therefore their bioavailability, is still partially unclear. Borges *et al.* postulate, that anthocyanins are poorly absorbed and pass the small to the large intestine where they are degraded by the colonic bacteria (*28*).

The proportion of the administered dose of anthocyanins not found in the ileostomy bags, that could have been absorbed, metabolized, and rapidly conjugated to sulfates or glucuronides, as shown by other groups (*34*, *56–58*), is still not known. Deglycosylation and rapid degradation via ring fission could occur. Absorption of anthocyanins might be low, and intensive degradation or metabolization must be considered. Nevertheless, anthocyanins reaching the colon are known to contribute to the prevention of inflammatory diseases; but precisely which active compounds promote positive health benefits is still unclear and they need yet to be identified. The results in the present paper show that most of the bilberry anthocyanins are recovered in the ileostomy effluent. While the five volunteers that took part in this study do not form a statistically rigorous sample, the information on the colonic availability of anthocyanins is confirmed. These identified compounds would reach the colon under normal physiological circumstances and there contribute to the prevention of inflammatory bowel diseases (IBD) and other malignant gastrointestinal diseases.

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References

1. Clifford, M. N. *J. Sci. Food Agric.* **2000**, *80*, 1063–1072.
2. Iwashina, T. *J. Plant Res.* **2000**, *113*, 287–299.
3. Manach, C.; Scalbert, A.; Morand, C.; Rémésy, C.; Jiménez, L. *Am. J. Clin. Nutr.* **2004**, *79*, 727–747.
4. Cheynier, V. *Am. J. Clin. Nutr.* **2005**, *81*, 223S–229S.
5. Harborne, J. B.; Williams, C. A. *Phytochemistry* **2000**, *55*, 481–504.
6. Scalbert, A.; Williamson, G. *J. Nutr.* **2000**, *130*, 2073S–2085S.
7. Jackman R. L.; Smith J. L. In *Natural Food Colorants*; Hendry, G. A. F., Houghton, J. D., Eds.; Springer: London, Glasgow, 1996.
8. Prior, R. L.; Lazarus, S. A.; Cao, G.; Muccitelli, H.; Hammerstone, J. F. *J. Agric. Food Chem.* **2001**, *49*, 1270–1276.
9. Kalt, W.; Ryan, D. A. J.; Duy, J. C.; Prior, R. L.; Ehlenfeldt, M. K.; VanderKloet, S. P. *J. Agric. Food Chem.* **2001**, *49*, 4761–4767.
10. Bermúdez-Soto, M. J.; Tomás-Barberán, F. A. *Eur. Food Res. Technol.* **2004**, *219*, 133–141.
11. Mazza, G.; Kay, C. D.; Cottrell, T.; Holub, B. J. *J. Agric. Food Chem.* **2002**, *50*, 7731–7737.
12. Kay, C. D.; Holub, B. J. *Br. J. Nutr.* **2002**, *88*, 389–398.
13. Kaehkoenen, M. P.; Heinämäki, J.; Ollilainen, V.; Heinonen, M. *J. Sci. Food Agric.* **2003**, *83*, 1403–1411.
14. Rice-Evans, C. A.; Miller, N. J. *Biochem. Soc. Trans.* **1996**, *24*, 790–795.
15. Robards, K.; Prenzler, P. D.; Tucker, G.; Swatsitang, P.; Glover, W. *Food Chem.* **1999**, *66*, 401–436.
16. Scalbert, A.; Johnson, I. T.; Saltmarsh, M. *Am. J. Clin. Nutr.* **2005**, *81*, 215S–217S.
17. Satué-Gracia, T.; Heinonen, M.; Frankel, E. N. *J. Agric. Food Chem.* **1997**, *45*, 3362–3367.
18. Goyarzu, P.; Malin, D. H.; Lau, F. C.; Taglialatela, G.; Moon, W. D.; Jennings, R.; Moy, E.; Moy, D.; Lippold, S.; Shukitt-Hale, B.; Joseph, J. A. *Nutr. Neurosci.* **2004**, *7*, 75–83.
19. Atalay, M.; Gordillo, G.; Roy, S.; Rovin, B.; Bagchi, D.; Bagchi, M.; Sen, C. K. *FEBS Lett.* **2003**, *544*, 252–257.
20. Olsson, M. E.; Gustavsson, K.-E.; Andersson, S.; Nilsson, A.; Duan, R.-D. *J. Agric. Food Chem.* **2004**, *52*, 7264–7271.
21. Cooke, D.; Schwarz, M.; Boocock, D.; Winterhalter, P.; Steward, W. P.; Gescher, A. J.; Marczylo, T. H. *Int. J. Cancer* **2006**, *119*, 2213–2220.
22. Simmen, F.; Xiao, R.; Wu, X.; Prior, R. *FASEB J.* **2008**, *22*, 887.
23. Kresty, L. A.; Morse, M. A.; Morgan, C.; Carlton, P. S.; Lu, J.; Gupta, A.; Blackwood, M.; Stoner, G. D. *Cancer Res.* **2001**, *61*, 6112–6119.
24. Weisel, T.; Baum, M.; Eisenbrand, G.; Dietrich, H.; Will, F.; STockis, J.-P.; Kulling, S.; Ruefer, C.; Johannes, C.; Janzowski, C. *Biot. J.* **2006**, *1*, 388–397.
25. Spormann, T.; Albert, F.W.; Rath, T.; Dietrich, H.; Will, F.; Stockis, J.-P.; Eisenbrand, G.; Janzowski, C. *Cancer Epidemiol. Biomarkers Prev.* **2008**, *17*, 3372–3380.

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Publication Date (Web): March 18, 2010 | doi: 10.1021/bk-2010-1035.ch010

Downloaded by UNIV OF DELAWARE MORRIS LIB on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): March 18, 2010 | doi: 10.1021/bk-2010-1035.ch010

26. *Hagers Handbuch der pharmazeutischen Praxis*, 4th ed.; Hager, H., List, P. H., Eds.; Springer: Berlin.
27. He, J.; Magnuson, B. A.; Giusti, M. M. *J. Agric. Food Chem.* **2005**, *53*, 2859–2866.
28. Borges, G.; Roowi, S.; Rouanet, J.-M.; Duthie, G. G.; Lean, M. E. J.; Crozir, A. *Mol. Nutr. Food Res.* **2007**, *51*, 714–725.
29. Coleman, J.; Wrzosek, T.; Roman, R.; Peterson, J.; McAllister, P. *J. Chromatogr., A* **2001**, *917*, 23–27.
30. Wu, X.; Prior, R. *J. Agric. Food Chem.* **2005**, *53*, 3101–3113.
31. Kahle, K.; Kraus, M.; Scheppach, W.; Ackermann, M.; Ridder, F.; Richling, E. *Mol. Nutr. Food Res.* **2006**, *50*, 418–423.
32. Goiffon, J.-P.; Mouly, P. P.; Gaydou, E. M. *Anal. Chim. Acta* **1999**, *382*, 39–50.
33. Sellapan, S.; Akoh, C. C.; Krewer, G. *J. Agric. Food Chem.* **2002**, *50*, 2432–2438.
34. Fleschhut, J.; Bub, A.; Rechkemmer, G.; Winterhalter, P.; Kulling, S.E. Proceedings of the XXII Conference on Polyphenols, Helsinki, Finland, 2004; PL12, pp 23−24.
35. Wu, X.; Cao, G.; Prior, R. L. *J. Nutr.* **2002**, *132*, 1865–1871.
36. Felgines, C.; Talavéra, S.; Gonthier, M.-P.; Texier, O.; Scalbert, A.; Lamaison, J.-L.; Rémésy, C. *J. Nutr.* **2003**, *133*, 1296–1301.
37. Mazza, G.; Kay, C. D.; Cottrell, T.; Holub, B. J. *J. Agric. Food Chem.* **2002**, *50*, 7731–7737.
38. Frank, T.; Netzel, M.; Strass, G.; Bitsch, R.; Bitsch, I. *Can. J. Physiol. Pharmacol.* **2003**, *81*, 423–435.
39. Frank, T.; Janssen, M.; Netzel, M.; Straß, G.; Kler, A.; Kriesl, E.; Bitsch, I. *J. Clin. Pharmacol.* **2005**, *45*, 203–210.
40. McGhie, T. K.; Ainge, G. D.; Barnett, L. E.; Cooney, J. M.; Jensen, D. J. *J. Agric. Food Chem.* **2003**, *51*, 4539–4548.
41. Cao, G.; Muccitelli, H. U.; Sánchez-Moreno, C.; Prior, R. L. *Am. J. Clin. Nutr.* **2001**, *73*, 920–926.
42. Matsumoto, H.; Inaba, H.; Kishi, M.; Tominaga, S.; Hirayama, M.; Tsuda, T. *J. Agric. Food Chem.* **2001**, *49*, 1546–1551.
43. Han, C.; Ding, H.; Casto, B.; Stoner, G. D.; D’Ambrosio, S. M. *Nutr. Cancer* **2005**, *51*, 207–217.
44. Harris, G. K.; Gupta, A.; Nines, R .G.; Kresty, L. A.; Habib, S. G.; Frankel, W. L.; LaPerle, K.; Gallaher, D. D.; Schwartz, S. J.; Stoner, G. D. *Nutr. Cancer* **2001**, *40*, 125–133.
45. Aura, A.-M.; Vainionpää, M.; Heinonen, M.; Lopéz, P. M.; Santos-Buelga, C.; Oksman-Caldentey, K.-M.; Puupponen-Pimiä, R.; Poutanen, K. S. Proceedings of the XXII Conference on Polyphenols, Helsinki, Finland, 2004; P9, p 121.
46. Keppler, K.; Humpf, H.-U. *Bioorg. Med. Chem.* **2005**, *13*, 5195–5205.
47. Fleschhut, J.; Kratzer, F.; Rechkemmer, G.; Kulling, S. E. *Eur. J. Nutr.* **2005**, *45*, 7–18.
48. Nurmi, T.; Mursu, J.; Heinonen, M.; Nurmi, A.; Hiltunen, R.; Voutilainene, S. *J. Agric. Food Chem.* **2009**, *57*, 2274–2281.

In Flavor and Health Benefits of Small Fruits; Qian, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

49. Hollman, P. C. H.; De Vries, J. H. M.; Van Leeuwen, S. D.; Mengelers, M. J. B.; Katan, M. B. *Am. J. Clin. Nutr.* **1995**, *62*, 1276–1282.
50. Walle, T.; Otake, Y.; Walle, K.; Wilson, F. A. *J. Nutr.* **2000**, *130*, 2658–2661.
51. Manach, C. *Am. J. Clin. Nutr.* **1995**, *62*, 1276–1282.
52. Olthof, M. R.; Hollman, P. C. H.; Katan, M. B. *J. Nutr.* **2001**, *131*, 66–71.
53. Kahle, K.; Kraus, M.; Scheppach, W.; Richling, E. Colonic availability of apple polyphenols: A study in ileostomy subjects. *Mol. Nutr. Food Res.* **2005**, *49*, 1143–1150.
54. Knaup, B.; Kahle, K.; Erk, T.; Valotis, A.; Scheppach, W.; Schreier, P.; Richling, E. *Mol. Nutr. Food Res.* **2007**, *51*, 1423–1429.
55. Eckburg, P. B.; Bik, E. M.; Bernstein, C. N.; Purdom, E.; Dethlefsen, L.; Sargent, M.; Gill, S. R.; Nelson, K. E.; Relman, D. A. *Science* **2005**, *308*, 1635–1638.
56. Passamonti, S.; Vrhovsek, U.; Vanzo, A.; Mattivi, F. *FEBS Lett.* **2003**, *544*, 210–213.
57. Kay, C. D.; Mazza, G.; Holub, B. J.; Wang, J. *Br. J. Nutr.* **2004**, *91*, 933–942.
58. Wu, X.; Pittman, H. E.; Prior, R. L. *J. Nutr.* **2004**, *134*, 2603–2610.

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Chapter 11

Cranberry and Grape Juice Drinks Affect Infectivity, Integrity, and Pathology of Enteric Viruses in an Animal Model

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The health benefits of potable fruit juices prepared from species of grape (e.g., *Vitis labrusca*) and cranberry (*Vaccinium macrocarpon*) have been the focus of numerous anecdotal and prospective studies. Few investigators have addressed the antiviral activity of juices from these or related species on enteric (intestinal) viruses within the family Reoviridae. Simian rotavirus SA-11 and bovine reovirus type 3 were used in these studies as representative enteric viral agents. Infectivity titers/viral detection were determined by isolation/immunofluoresence, transmission electron microscopy (TEM), polyacrylamide gel electrophoresis (PAGE) of viral dsRNA, and the reverse transcription polymerase chain reaction (rtPCR). Pretreatment of monolayers of monkey kidney epithelial-like (MA-104) cells in culture with either store-purchased or manufacturer-supplied (no added sugar) cranberry (CJ) and grape juice (GJ) at concentrations $\geq$ 16% of control reduced infectivity titers of rota- and reovirus by

In Flavor and Health Benefits of Small Fruits; Qian, M., et al.;
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more than one order of magnitude. Vitamin C at concentrations present in store-purchased juice drinks had no effect on viral titers. Reovirus dsRNA was not detected by PAGE in monolayers pretreated with either juice and in cell-free suspensions of virus, suggesting an adverse effect by both juices on adsorption/penetration and a direct inactivation of viral particles *per se*. TEM did not detect virus penetration or egress among monolayers pretreated with store-purchased or manufacturer-supplied CJ. Detection of rotavirus RNA (i.e., amplicon yield) by rtPCR was markedly reduced after pretreatment of monolayers with manufacturer-supplied CJ and GJ drinks. Loss of cell viability/cytotoxicity of the host cells as a result of exposure to the juices was not observed by trypan blue exclusion, cell passage, or quantitative adenylate kinase release. Antiviral activity by CJ and GJ drinks *in vitro*, was supported by testing in the mouse model.

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Introduction

Antiviral drugs are continually being developed to meet the challenge of emerging, reemerging, and current viral infections. However, toxicity and the enrichment of resistant strains continue to plague the development and use of such drugs. Vaccines are available against numerous viral groups, but, especially in developing countries, issues of production and delivery (e.g., rotavirus vaccine) to the populace persist (*1*, *2*).

The health benefits of "medicinal" phytochemicals (e.g., flavonols and flavan-3-ols) and potable fruit juices prepared from, for example, cranberry (*Vaccinium macrocarpon*) or grape (*Vitis labrusca*), have been the focus of numerous studies (*3–6*). *In vitro* and *in vivo* studies have suggested improved health benefits through the consumption/administration of potable juices or their extracts/concentrates on, for example, cholesterol levels, cardiac circulation after ischemia, atherosclerosis, the inflammatory response, periodontal disease, and urinary tract infections (*4*, *7–10*). The effects of cranberry juice/extracts as antiviral agents against herpes simplex virus, influenza virus, and more extensively, the human immunodeficiency virus type 1, have also been reported (*11–15*).

Intestinal viruses, especially rotaviruses, cause significant morbidity and mortality among infants and young children throughout the world. In developing countries, rotavirus-associated gastroenteritis is responsible for the deaths of over 500,000 infants and young children annually (*16*, *17*). In the United States, some 60,000 to 70,000 children are hospitalized with approximately 3 million cases of viral gastroenteritis each year. These numbers are responsible for hundreds of millions of dollars spent annually on health care. Although vaccines against strains of rotavirus are available and have shown efficacy in early studies, their use remains problematic due to side effects, "vaccine take" (viz., antibody response), and, in developing countries, cost, host, and environmental issues (e.g., interference by maternal antibodies, other microbial infections, malnutrition) (*1*, *2*). Furthermore, vaccines against other intestinal viruses (e.g., enteroviruses, caliciviruses), where treatment is supportive are not available. A need exists to explore other avenues to reduce or ameliorate enteric viral diseases. Health benefits by the consumption of comestible plant products (e.g., cranberry juice, grape juice) might be a natural mode in the ever-present battle against viruses and other microbial diseases (*3*, *7*, *18*, *19*).

Our studies have shown antiviral effects on bacteriophages and mammalian intestinal viruses of cranberry and grape juices (*20–22*). However, few studies have addressed the mechanism(s) responsible for the inhibitory or inactivating effects of CJ and GJ on enteric (intestinal) viruses. Here, we review earlier and recent findings of the mechanisms associated with the antiviral activity of CJ and GJ.

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Materials and Methods

Viruses and Cell Culture

Bovine reovirus type 3 and rotavirus strain SA-11 were obtained from American Bioresearch Laboratories (ABL; Sevierville, TN) and the American Type Culture Collection (VR-1565), respectively. The viral host was of African green monkey kidney, clone *Ceropithecus aethips* epithelial-like (MA-104) cells which were obtained from ViroMed Laboratories (Minneapolis, MN). Growth and storage of virus and cell culture techniques were performed as previously described (*22*, *23*).

Immunofluorescence

Polyclonal antibodies, tagged with fluorescein isothiocyanate (FITC), to reovirus and rotavirus were supplied by ABL and Genway Biotech, Inc. (San Diego, CA), respectively. Monolayers of MA-104 cell cultures, supplied in 1- dram glass shell vials with snap-top caps (ViroMed Laboratories, Inc., Minneapolis, MN) were immunostained with the appropriate antibody to early viral antigens, incubated overnight, and then harvested as described previously (*22*, *23*). Infectivity titers were reported as fluorescent focus units (FFUs) /ml and reported as a per cent of control.

Effect of Juices on Viral Infectivity Titers

Monolayers of MA-104 cell cultures were inoculated with 0.2 ml CJ and GJ before (pretreatment) or after (posttreatment) with virus. Both store-purchased and manufacturer-supplied pure juices were utilized in these studies. Manufacturer-supplied juices consisted of Cranberry Juice Drink and

“Concord” (purple) and “Niagara” grape-juices [Ocean Spray Cranberries, Inc. (Lakeville-Middleboro, MA) and Welch Foods, Inc. (Concord, MA), respectively]. Manufacturer-supplied juices were not supplemented with sugar or vitamin C. Cranberry Juice Cocktail drink from Concentrate (store-purchased CJ, Ocean Spray) and Welch’s 100% Grape Juice From Concentrate with Vitamin C, “From Welch’s Own Concord Grapes” (store-purchased GJ; Welch Foods) were purchased from a local food store. Pretreatment studies were performed using HPLC-extracted cranberry high molecular weight (CB HMW) and low molecular weight (CB LMW) proanthocyanidins (PACs), CB anthocyanidins (CB-CA), cranberry sodium acetate (CB EtoAC), and grape (100% grape juice from concentrate) PAC (GP-PAC) extracts (kindly supplied by Dr. A. B. Howell, Rutgers University; (*8*)). Working strength juices/analytes were filter-sterilized (0.2 um filter) before use.

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Pretreatment of Monolayers

Briefly, noninfected monolayers of MA-104 cells grown in 1-dram glass vials were treated with 0.2 ml of increasing juice or analyte (e.g., proanthocyanidins) juice concentrations for 3 to 5 minutes and room temperature (23°C). Monolayers were washed with phosphate-buffered saline (PBS) without Ca^{2+} or Mg^{2+} and inoculated with 0.2 ml of reovirus or rotavirus. After incubation for 50 to 60 min at 37°C, the monolayers were washed with PBS, refed with cell culture maintenance medium (*23*), and incubated for an additional 16 to 24 h at 37°C. Cultures were harvested and immunostained with the appropriate FITC-tagged antibody to determine infectivity titers. All experiments were performed in triplicate and expressed as means +/- standard errors of the means. Stock viral titer consisted of 2 X 10^6 FFU/ml.

Posttreatment of Monolayers

Monolayers of MA-104 cells were inoculated with 0.2 ml of rotavirus, incubated at 37°C for 50 to 60 min, washed with PBS, treated with 0.2 ml of increasing concentrations of manufacturer/store-purchased cranberry juices, and “Concord” (purple or “Niagara”-grape) juices for 3 to 5 min. at room temperature, washed with PBS, refed, incubated overnight (16 to 24 h), and immunostained to determine infectivity titers, as described above. Experiments were performed in triplicate and results expressed as means +/- standard errors of the means. Viral stock titer: 2 X 10^6 FFU/ml.

Determination of Cell Viability/Cytotoxicity.

Cell viability/cytotoxicity was initially evaluated by Trypan Blue exclusion, as described previously (*22*). Briefly, 25% concentrations of store-purchased CJ or GJ drinks in PBS were added to MA-104 cultures in T25-cm^2 flasks for 3 to 5

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min at room temperature. Juices were removed by aspiration and the cells washed with PBS. 5 ml of a (0.4% solution of Trypan Blue reagent (Sigma-Aldrich, St. Louis, MO) was added to equal volumes of trypsinized cell suspensions. Viable (i.e., unstained) cells were examined for morphological aberrancy (e.g., cytoplasmic projections; (*24*)). The control consisted of monolayers treated with only PBS. Cell growth was determined after treatment of monolayers. Briefly, monolayers were pretreated with stored-purchased GJ drink, as described above, subpassaged, and maintained for a period of 9 days in culture (with appropriate changes of medium), followed by trypsinization and cell quantitation using a Neubauer hemocytometer (*25*). Control cultures were treated exactly as the experimentals, but treated only with PBS. The ToxiLight^R BioAssay (Cat. No. LT17-217; Lonza Rockland, Inc., Rockland, ME), a nondestructive bioluminescent cytotoxicity assay, was used to measure quantitatively the release of adenylate kinase (AK) from potentially damaged cells in monolayer culture. MA-104 monolayers were washed with PBS, followed by the addition of 10% suspensions of manufacturer-supplied GJ and CJ. Cytotoxicity/cellular integrity was measured by release of AK, using bioluminescence as a marker system), as per manufacturer's instructions. Emitted light was measured with a beta counter (Model LS6000LL; Beckman Instruments, Inc., Fullerton, CA) set to a read-time of 1 sec. Readings were expressed as counts per min. The negative and positive controls consisted of monolayers treated only with PBS or ToxiLight^R 100% Lysis Reagent (Cat. No. LT07-517; Lonza Rockland, Inc.), respectively. Experiments were performed in triplicate and results expressed as means +/- error of the means.

Transmission Electron Microscopy (TEM)

Confluent MA-104 monolayers in T25-cm^2 flasks were inoculated with 1 ml of rotavirus SA-11 (2 X 10^6 FFU/ml), incubated for 50 to 60 min at 37°C, washed with PBS, and then treated with 1 ml of manufacturer-supplied CJ for 3 to 5 min at room temperature. The monolayers were washed with PBS, refed, and incubated for 72 h at 37°C. After the 72-h incubation, the monolayers were washed with PBS, scraped, fixed with 3% glutaraldehyde in 0.2 M sodium cacodylate buffer, treated with 1% osmium tetroxide, dehydrated in graded ethanol, embedded in epon, ultrathin sectioned, and mounted on grids for examination by TEM (*26*, *27*).

Polyacrylamide Gel Electrophoresis (PAGE) Analysis of Viral dsRNA; Determination of Viral Integrity

Virus-juice suspensions were prepared to determine whether manufacturer-supplied CJ and store-purchased GJ were capable of directly affecting loss of viral integrity. The reovirus was used in these studies. Briefly, 2 ml of an undiluted stock suspension of virus (2 and 10^6 FFUs/ml) were added to an equal volume of 10% CJ and GJ in PBS that had been filter-sterilized (0.2-um filter) to eliminate bacterial contamination. After 30 or 60 min of incubation at room temperature, suspensions were concentrated (polyacrylamide absorbent gel; Cat. No. 81128;

Sigma-Aldrich, St. Louis, MO), followed by extraction of viral RNA [AquaPure RNA isolation kit (Cat. #732-6370; BioRad Laboratories, Hercules, CA)], as per manufacturer's instructions. The RNA was suspended in Laemli sample buffer and loaded onto polyacrylamide gels (Precast PAGEr 4-12% T-G) for analysis of the viral genomic pattern. RNA segments were visualized by silver staining (BioRad Kit No. 161-0449; (*28*)).

Reverse Transcription Polymerase Chain Reaction (rtPCR)

MA-104 cells were grown to confluency in T25-cm^2 flasks, treated for 3 to 5 min at room temperature with 50% concentrations (in PBS) of manufacturer-supplied CJ (100% Cranberry juice drink without vitamin C) and Concord (purple) grape juice, followed by aspiration and washing with PBS. The monolayers were inoculated with 1 ml volumes of rotavirus suspensions at concentrations of 2 X 10^6, 1 X 10^6, 2 X 10^5, 2 X 10^4 , and 2 X 10^3 FFU/ml and incubated at 37°C for 50 to 60 min. Total RNA was extracted using the Trizol RNA extraction kit (Cat. No. 15596-026; Invitrogen, Carlsbad. CA), as per manufacturer's specifications. RNA (1 ug) from each sample was used for a two-step rtPCR using superscript II reverse transcriptase (Cat. No. 18064-014; Invitrogen). The PCR was performed using TAQ polymerase (5 ul/50 ul of reaction mixture) (Invitrogen; Cat. No. 10342-020). Five ul were used per 50 ul of reaction mixture. The PCR cycle conditions consisted of step 1 at 94°C for 2 min (denaturation), step 2 at 94°C for 30 sec (denaturation), step 3 at 55°C for 30 sec (annealing), and step 5 at 72°C for 7 min (extension). Steps 2 to 4 were repeated 35 times. Beginning and end "primer 9 sequences" were 5'- GGC TTT AAA AGA GAG AAT TTC CGT CTG G – 3' and 5' – GGT CAC ATC ATA CAA TTC TAA TCT AAG – 3', respectively (*29*). The PCR products (1078-bp fragments) were analyzed on a 2% agarose gel containing 0.5 ug ethidium bromide per ml. After verifying the products, 4 ul from each amplicon was subjected to a second PCR round of amplification using the same conditions and primers.

Virus Infectivity Testing in the Mouse Model

All mouse studies which used CD-1 (Crl:CD-1-nuBR) T-cell deficient female nude mice (Charles River Laboratories, Wilmington, M) were approved by the University Animal Welfare Committee (UAWC; New York University - Washington Square Campus, New York, NY). A T-cell deficient mouse strain was chosen for this phase of the study to control for a potential masking of results by the animals' immune response. The testing of store-purchased CJ (cocktail0 and GJ drinks in mice was performed as follows: Briefly, 0.2 ml of virus plus store-purchased CJ or GJ drinks (40% juice concentration at a 1:1 juice-virus ratio) were orally introduced into mice by gavage. The positive control consisted of virus plus PBS. The negative control consisted of PBS alone. Noninoculated mice were maintained during the experimental period to eliminate the possibility of a confounding effect by endogenous or exogenous pathogens. All mice

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experiments were performed in pentuplicate. Upon mortality of the positive control mice, experimentals, negative controls, and noninoculated mice were euthanized with 100% CO_2 and examined by dissection and colon histology. Distal-colon sections were placed into 10% neutral buffered formalin, dehydrated in a graded series of ethanol, placed into xylene, embedded in paraffin, sectioned (5 um), rehydrated in graded ethanol solutions, and stained with hematoxylin and eosin (*30*). The tissue specimens were analyzed at magnifications of 100, 200, and 400X. Colon specimens for viral isolation testing were placed into PBS supplemented with 2% fetal bovine serum (FBS) and frozen at -50°C before testing.

Statistics

Cell culture-based experiments were performed in triplicate or quadruplicate. Immunofluorescence readings were performed by at least three individuals. The data are presented as the arithmetic means +/- standard error of the mean. Control and experimental means were compared by the Student's "t", test where $P < 0.05$ was assumed to be statistically significant.

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Results and Discussion

Pretreatment of monolayers of MA-104 cells with manufacturer-supplied CJ and GJ reduced reovirus infectivity titers by >90% at [juice] concentrations of 16 to 30% (Fig. 1). Similar declines in infectivity titers of rotavirus occurred on MA-104 monolayers pretreated with manufacturer-supplied and store-purchased CJ drinks (Fig. 2). There was no synergistic effect with 3% concentrations of each store-purchased CJ and GJ drinks (*20*, *22*). TEM showed an inhibition or arrest of reovirus entry into the normally permissive MA-104 host cells that had been pretreated with CJ cocktail drink, whereas morphologically recognizable reovirus particles were observed in control cultures pretreated with only PBS (*21*).

Neither store-purchased grape nor CJ cocktail drinks caused a cytotoxic effect on the culture monolayers (Fig. 3). These data suggest an inhibition or blockage by the juice drinks of the entry of reo- and rotavirus into the host cell. Furthermore, recognized differences in viral antigenic determinants (ligands) and host cells receptor sites in the reovirus/rotavirus-host cell systems suggest that the antiviral activity of CJ and GJ antiviral activity is nonspecific (*31*–*33*).

Studies were conducted to determine whether, and which, specific chemicals in CJ or GJ drinks might be responsible for the reductions in viral infectivity titers. Thousands of phenolic metabolites, including flavonoids, exist in plants, with possibly hundreds of these compounds present in species of cranberry and grape (*3*, *5*, *13*, *33*–*35*). Consequently, experimentation was restricted to flavonoids that had been shown in earlier studies to have antimicrobial activity (*7*, *8*, *36*). For example, a proanthocyanidin (PAC) [primarily type A] extract of cranberry was shown to be a potent inhibitor of the attachment of P-fimbriated *Escherichia*

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coli to human uroepithelial cells, presumably as a result of the blockage of the binding to receptor sites on host cells (*8*, *37–39*). The potential antiviral activity of cranberry and grape PACs and other selected extracts (e.g., anthocyanidins) in our virus-cell system was evaluated. Pretreatment studies showed reduced reovirus infectivity titers in the order GP PAC > CB low molecular weight PAC (CB LMW) > CB high molecular weight PAC (CB HMW) > CB sodium acetate (CB EtoAC) (Fig. 4), which reflected the immisciblity of CB HMW PAC in PBS. High speed homogenization of the immiscible CB HMV PAC fraction produced a CB product that was as effective as GP PAC (*22*). This finding parenthetically, accentuates the importance of the bioavailability or delivery of the flavonoid to the virus-cell assay systems. The soluble CB anthocyanidin extract had no effect on the reduction of viral infectivity titers. Differences, as well as similarities, in antiviral activity among the various extracts used might have been the result of the uniquely inherent chemical structures of flavonoids, which are characteristic of plant secondary metabolites (*4*, *5*, *13*, *39*, *40*). Antiviral activity of store-purchased fruit juice drinks, grape extracts (e.g., of *Vitis vinifera, V. labrusca*), and wines (red, white, rose), against enteroviruses (polio-, coxsackie-, and echoviruses), a herpes virus type I, and reovirus type 1 (wines only), have been investigated (*41*, *42*). Antiviral activity generally occurred in the order, grape > apple > cranberry > tea drinks. Wines had variable activity against the reo- and other viruses. A mechanistic interpretation of these data is difficult, as these antiviral activity studies were performed only with cell-free viral suspension systems and with analytes characterized only by differential filtration. However, these data suggested a relation between loss of infectivity titer and increasing molecular weight of the filtrates.

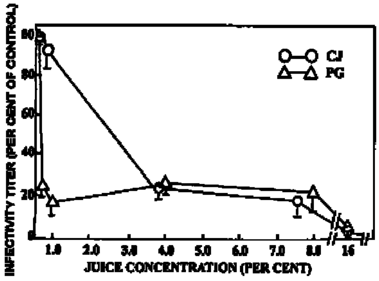

Figure 1. Effect of MA-104 cell monolayer pretreatment by manufacturer-supplied cranberry juice (CJ) and Concord (purple) grape juice (PG) drinks on the infectivity titer of reovirus.Monolayers were pretreated with increasing concentrations of each juice. After 3-5 min., monolayers were washed and subsequently immunostained to detect viral antigen. Titers were expressed as flourescent focus units (FFUs)/ml. Viral stock titer: 2 X 10^6 *FFUs/ml.*

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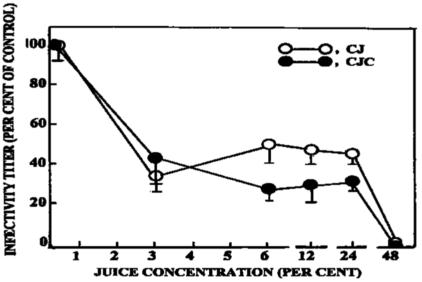

Figure 2. MA-104 epithelial cell culture monolayers pretreated with different concentrations of manufacturer-supplied cranberry juice (CJ) and store-purchased cranberry juice cocktail (CJC) drinks on infectivity titers of rotavirus. Virus stock titer: 2 X 10^6 FFUs/ml.

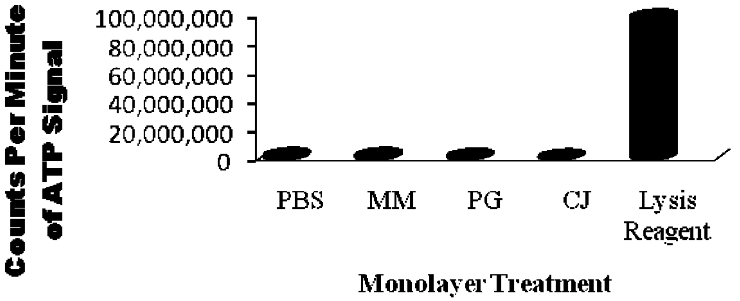

Figure 3. Effect of manufacturer-supplied cranberry juice (CJ) and Concord (purple) grape juice (PG) on cell viability. Cell monolayers were pretreated with 10% CJ and PG juices for 3-5 min, followed by the measurement of adenylate kinase release. PBS, Phosphate-buffered saline; MM, Manitenance medium.

Manufacturers commonly add ascorbic acid (vitamin C) to juice drinks or juice beverages. The "Cranberry Cocktail Juice Drink" and the "Welch's 100% Grape Juice from Concentrate with Vitamin C" used in some of our studies are fortified with a 100% vitamin C daily value as declared in each Nutrition Facts Panel as per federal standards (see: United States Department of Agriculture Code of Federal Regulation (21 CFR 101.9). Consequently, testing was performed to identify whether ascorbic acid (kindly supplied by Welch Foods, Inc.) might effect the measurement of viral infectivity titers in host cell cultures. Ascorbic acid, at levels present in store-purchased CJ or GJ drinks, had no modifying effects on reovirus infectivity titers (not shown; (*28*, *35*)).

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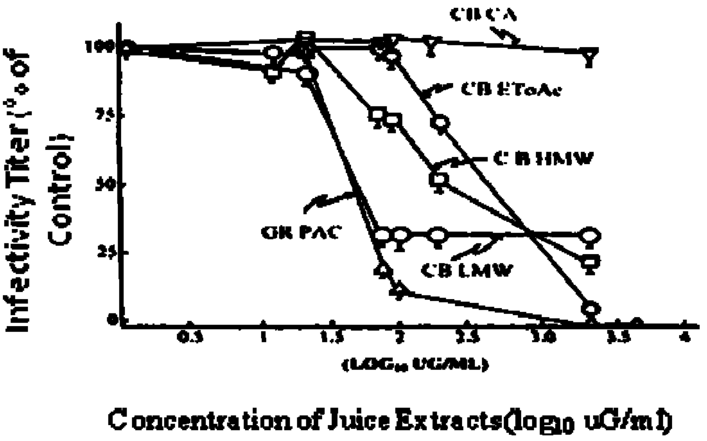

Figure 4. Infectivity titers of reovirus after pretreatment of MA-104 cell monolayers with proanthocyanidins (PAC) and other plant extracts. CB-HMW, cranberry high molecular weight PAC; CB-LMW, cranberry low molecular weight PAC; CB-CA, cranberry anthocyanidins; CB EtoAC, cranberry sodium acetate; GR-PAC, Welch's 100% Grape Juice PAC from concentrate. Means +/- standard error of the means. (Reproduced with permission from reference (22). (Copyright 2007 Wiley-VCH Verlag GmbH & Co.)

In an effort to define potential mechanisms of manufacturer-supplied cranberry juice drink on the inhibition or reduction of enteric virus infectivity titers, monolayers were subjected to posttreatment regimens employing another intestinal viral agent, rotavirus strain SA-11. Cellular infection was monitored by TEM over 30-, 60-, and 90- min viral adsorption periods in order to visualize morphologic events after juice treatment of cells. During 30- and 60-min viral adsorption periods, no entry was observed og rotavirus, similar to what has been reported in reovirus infected monolayers (*21*). However, after a 90 min adsorption period, entry of the rotavirus into host epithelial cells occurred, but particle movement through the cell was arrested at the level of cellular cisternae. Positive control cultures (i.e., rotavirus plus only PBS), morphologically viral recognizable particles accumulated in the cytosol and at the inner surface of the plasma membrane before and during egress (Fig. 5). The results of these ultrastructural studies were supported by infectivity titer assays, where CJ concentrations of 48% reduced levels of rotavirus by > two orders of magnitude (Fig. 2).

The isolation/detection of virus in cell culture, once considered the "gold standard", lacks the sensitivity of molecular assays. With the advent of the polymerase chain reaction (PCR) and, subsequently, rtPCR, detection of virus/viral RNA can be achieved at low levels not previously possible (*43*, *44*). Utilizing genomic amplification, we determined the minimal concentration of rotavirus to which CJ and GJ affect a reduction of particle yield in monolayer culture. Pretreatment of host cells with a 50% concentration of manufacturer-supplied CJ or GJ drinks showed a reduction in detection levels of rotavirus (i.e., amplicon yield) in the order 2 X 10^6 > 1 X 10^6 > 2 X 10^5 > 2 X 10^4 input FFU/ml (Fig. 6). The rotavirus RNA was not detected in monolayers pretreated with juices at virus input concentrations ≤ 2 X 10^4 FFU/ml. Increasing rotavirus concentrations (i.e., 2 X 10^6) in the rtPCR assay system was reflected by a concomitant obfuscation of the CJ- or GJ-associated antiviral effect. These results might have been ascribed by a combination of (1) increased cellular infection at higher particle concentrations and (2) the relatively ultrasensitive rtPCR assay. Specifically, virus attachment, and then irreversible binding and penetration into hosts cells in monolayer culture, are mediated by the physical forces of diffusion and Brownian motion (*45*). Increased virus concentration (viz., particles per unit volume), in concert with the physical forces of diffusion and Brownian motion, may have resulted in a greater efficiency of viral attachment and, in turn penetration through plasma membranes of CJ- or GJ- drink-pretreated cells. Genome amplification by rtPCR among treated and control cells in monolayer culture, and at increased viral input titers, failed to yield product bands of easily recognizable differential intensities. However, with reduced inocula titers introduced onto juice-treated cells in monolayer culture, the effect in virus particle blockage was significant, with easily recognizable differences in RNA banding intensities (Fig. 6). These data support, on the molecular, level the CJ- or GJ-associated inhibition of virus detected on the cellular level.

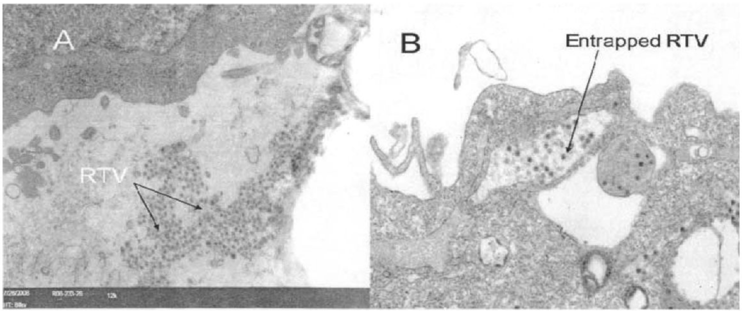

Figure 5. Transmission electron microscopy of rotavirus infected cell monolayers. Posttreatment by manufacturer-supplied cranberry juice (CJ) of rotavirus-infected monolayers. Cultures incubated 72-h. A, PBS-treated monolayer (positive control). Note dispersion of virus particles at cytoplasmic periphery. B, Treatment of monolayers with CJ. Note virus particles entrapped within cellular cisternae.

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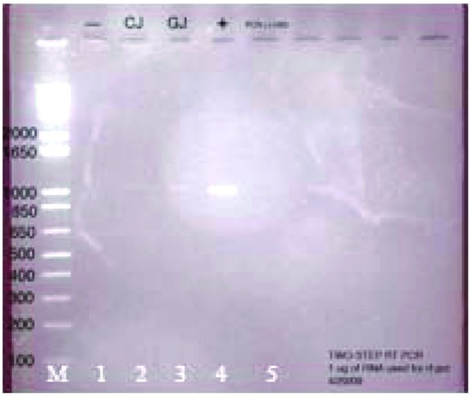

Figure 6. Amplification by rtPCR of the rotavirus 1078-bp RNA fragment. Monolayers of MA-104 cells were treated with 50% cranberry juice drink or Concord grape juice followed by incubation with 1 X 10^6 viral FFUs/ml. Lane M, 2000-bp ladder; Lane 1, RNA extract alone (negative control); Lane 2, RNA from cranberry juice-pretreated monolayer; Lane 3, Grape juice-pretreated monolayer; Lane 4, RNA from rotavirus in PBS (positive control); Lane 5 H_2O.

Posttreatment with manufacturer-supplied CJ and store-purchased CJ (cocktail) drinks resulted in similar reductions in monolayer infectivity titers of ca. 60% at concentrations of juice drinks of ca. 30% (Fig. 7). However, posttreatment by the juices was, in general, less effective in the reduction of viral infectivity titers than pretreatment. Manufacturer-supplied purple and Niagara grape juices reduced infectivity titers of rotavirus by ca. one order of magnitude at juice concentrations of 25% (Fig. 8). Similar reductions in viral titer curves by the Concord juice (containing higher levels of PACs) in comparison to Niagara juice suggest that PACs alone may not be ascribed to the antiviral antivity of these plant products.

The reduction in viral infectivity titers as a result of the treatment of monolayers with CJ and GJ drinks does not, in itself, denote a loss of viral structural integrity but, rather, suggests juice-associated modifications, albeit nonspecific, at the host cell surface. To determine whether CJ or GJ drinks directly affected viral infectivity or structural integrity in cell-free systems, the integrity of the reovirus particle in CJ and GJ drinks was determined by PAGE analysis of the viral RNA (*46*). Viral RNA was not detected by PAGE in the juice-virus cell-free suspensions (Figs. 9 and 10). Positive control suspensions (virus plus PBS) showed the typical RNA migratory pattern of the segmented genome of the reovirus. Loss of viral RNA integrity, determined by PAGE, in juice-pretreated cells in monolayer culture, reflected reductions in infectivity titration (*22*). Similar studies with viral-juice/wine systems were performed by Konowalchuk and Spears (*41*, *42*). However, determination of antiviral activity only by infectivity titration did not distinguish between a direct effect by the juices on virus integrity or on modifications of the surface of the host cell. Direct testing of juices on virus in cell-free suspension indicated, for the first time, a loss of viral particle integrity. It would not be unreasonable to suggest a possible disorganization of the protein viral capsid by CJ- and GJ-containing proanthocyanidins (viz., an astringency-associated effect) (*47–49*). The exposed viral genome would, in turn, be subject to lysis by omnipresent RNases.

Efficacy of the juices in reducing viral infectivity *in vitro* does not necessarily predict what will occur *in vivo*. Consequently, antiviral testing was extended to an animal (mouse) model. Clinical disease was absent in mice four days after oral administration of reovirus-CJ or reovirus-GJ drink preparations. Positive control mice, which were administered only virus in PBS, displayed systemic hemorrhage, loose stools/diarrhea, and dehydration, and they died 3 to 4 days after inoculation. Histological analysis of mouse colon revealed normal mucosa, intact goblet cells, and dense feces among animals treated with virus-CJ cocktail drink, whereas colon specimens from mice inoculated with virus-GJ drink suspensions displayed moderately shrunken mucosa, mucin-depleted goblet cells, and some inflammatory foci and debris in the lumen.

Markedly damaged mucosa with thinned muscularis externa was observed in positive (virus plus PBS) but not negative (only PBS) control mice (Fig. 11). Antiviral activity of flavonoids has been studied in the mouse model (*11*, *13*, *50*, *51*). Nagai and co-workers, for example, reported an inhibition of influenza A (H3N2) and influenza B activity in the respiratory tract of mice following pre- and post-oral administration of an extract of *Scutellara baicalensis* (Baikal Skullcap). The inhibitory effect was ascribed to a reduction of viral sialidase activity (*52*). The current mouse study, however, showed for the first time, a clinical protective effect by two common comestible juices to a mammalian enteric virus.

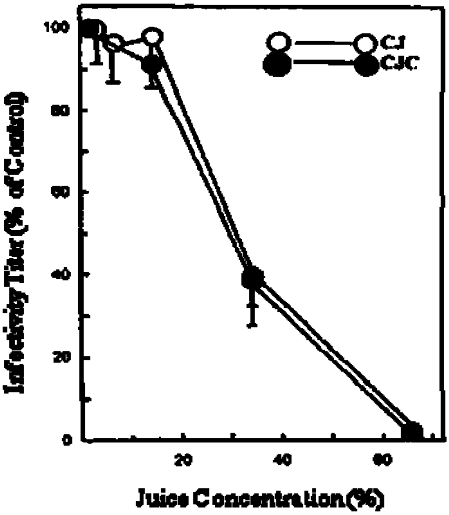

Figure 7. Infectivity titers of rotavirus after posttreatment of MA-104 cell cultures by manufacturer-supplied cranberry juice (CJ) and store-purchased cranberry juice cocktail (CJC) drinks. Virus stock titer: 2 X10^{6} FFU/ml. Means +/-standars error of the means.

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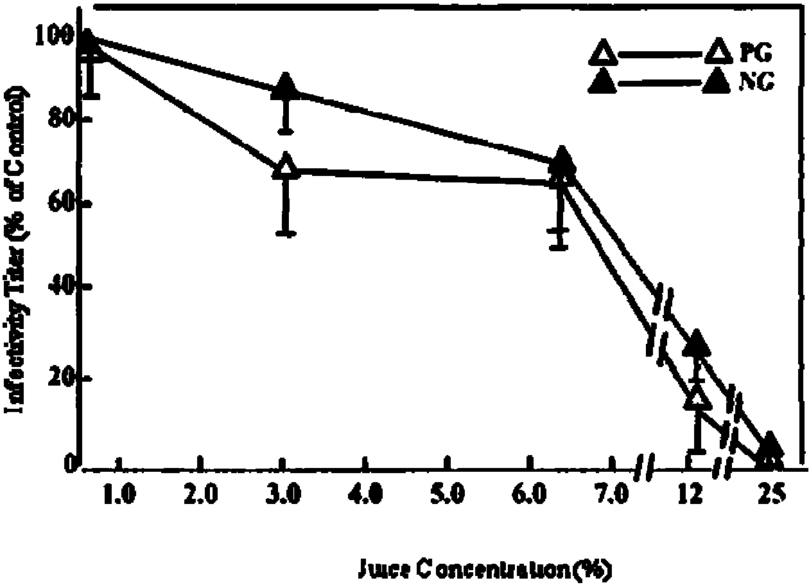

Figure 8. Infectivity titers of rotavirus after posttreatment of infected MA-104 monolayers by manufacturer-supplied Concord purple grape (PG) and Niagara grape (NG) juice drinks. Virus stock titer: 2 X 10^6 FFU/ml. Means +/-standars error of the means.

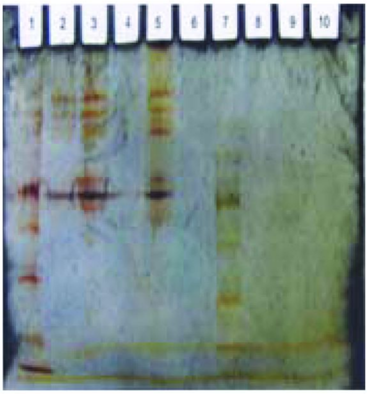

Figure 9. Effect of store-purchased cranberry juice cocktail (CJC) drink on the integrity of reovirus (RV) RNA. RV was added to equal volumes of CJC. After 30 min at 23°C, suspensions were prepared for PAGE analysis of the viral RNA. Lane 1,7: ProSieve^R 11-90 kDa protein marker; Lane 2,3,5: Positive control [RV (2 X 10^7 FFU/ml) + PBS]; Lane 4,6: Empty; Lane 8,9,10: RV (2 X10^7 FFU/ml) + CJC drink.

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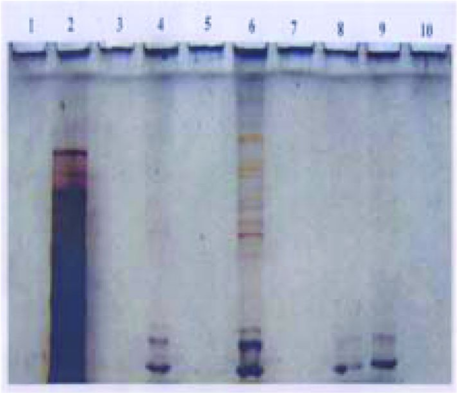

Figure 10. Effect of "100% Grape Juice from Concentrate with Vitamin C" on the integrity of reovirus (RV) genomic RNA.. RV was added to equal volumes of a 10% concentration of the grape juice (GJ) drink. After 60 min at 23°C, experimentals and controls were subjected to RNA extraction and PAGE analysis of the viral RNA. Lane 1,10: Empty; Lane 2: ProSieve^R 11-90 kDa protein marker; Lane 3,5,7: GJ + PBS; Lane 4: RV (2 X 10^4FFU/ml) + PBS; Lane 6: RV (2 X 10^7FFU/ml) + PBS; Lane 8,9: RV (2 X 10^7FFU/ml) + GJ.

Summary

Pretreatment with store-purchased and manufacturer-supplied cranberry and grape juices generally reduced reovirus and rotavirus infectivity titers in cell (MA-104) culture to levels ranging from no significant changes to more than one order of magnitude. Both store-purchased and manufacturer-supplied juices had similar antiviral effects. Based on the results of rtPCR and ultrastructural studies, CJ and GJ drinks appear to have reduced infectivity titers by an inhibition of viral penetration, although an inhibition of rotavirus egress, as demonstrated seen by particle sequestration at the level of cellular cisternae, may also have been involved. There are no data to support specific ligand-receptor site binding between the juices/plant extracts and animal viruses or mammalian cells. Posttreatment of monolayers with manufacturer-supplied CJ or GJ drinks reduced infectivity titers of rotavirus from 50 to >90% at juice concentration ≥30%. Cytotoxicity studies showed that infectivity titers were independent of nonviral-induced cellular destruction.

PAGE analyses of reovirus RNA (as a measure of viral integrity) of virus-juice suspensions in the absence of host cells showed no recognizable electrophoretic patterns, suggesting a direct inactivation/loss of viral infectivity by either CJ or GJ drinks.

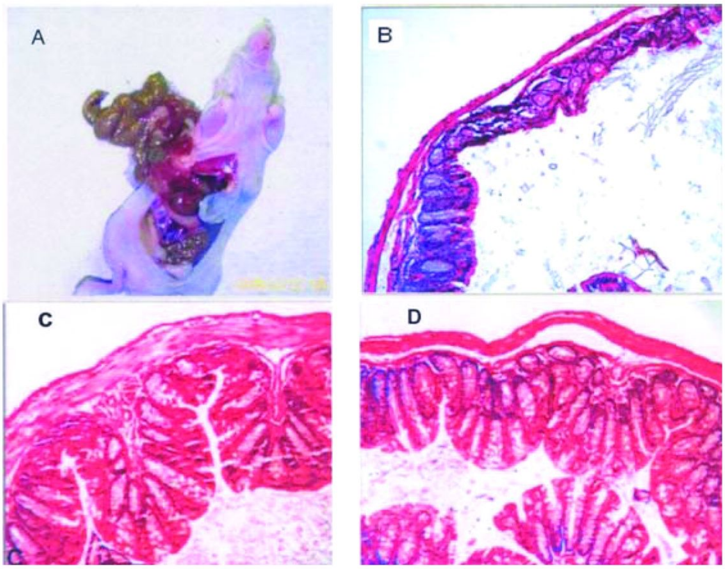

Figure 11. Histology of mouse colon: Antiviral activity of store-purchased cranberry juice (CJ) cocktail and Grape juice (GJ) drinks. Mice were inoculated with 40% juice-reovirus suspensions. Nine days p.i., mice were subjected to examination by autopsy. A, Positive control (autopsy). Virus-PBS inoculated mice presented with loose stool on autopsy. B, Positive control (histology). Mouse inoculated with PBS-virus suspension. Note severe damage to mucosa. Muscularis externa thinned in some areas; Distal colon histology among GJ-virus inoculated mice showed shrunken mucosa, mucin depleted goblet cells, large inflammatory foci, and debris in the lumen (not shown). C, Colon specimen prepared from mouse simultaneously inoculated with a 40% CJ-reovirus suspension. Note relatively intact goblet cells. D, Saline-inoculated mouse; Note normal mucosa (full height) and muscularis externa.

Studies in mice confirmed the efficacy of CJ and GJ drinks as antiviral agents observed in *in vitro* studies. These studies report, for the first time, an antiviral (viz., intestinal virus) effect of CJ or GJ drinks in an animal model.

Fruits and fruit juices, ostensibly safe and nutritious, are dietary staples throughout the world. Juice products manufactured from cranberry and grape account for a large portion of the world's beverage market. The antiviral activity utilizing reovirus and rotavirus as model enteric infectious agents, of these juices supplement the reported beneficial health aspects of CJ and GJ drinks (*7*, *18*). Antiviral activity of cranberry and grape juice drinks needs to be explored further, especially to define the role of these products in the clinical setting.

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References

1. *Principles of Virology: Molecular Biology, Pathogenesis, and Control*; Flint, S. J., Enquist, L. W., Krug, R. M., Racaniello, V. R., Skalka. A. M., Eds; ASM Press: Washington, DC, 2000; pp 663−670.
2. Parashar, U. D.; Glass, R. I. *New Eng. J. Med.* **2009**, *360*, 1063–1065.
3. Aron, M.; Kennedy, J. A. *Mol. Nutr. Food Res.* **2008**, *52*, 79–104.
4. Dixon, R. A.; Xie, D.-Y.; Sharma, S. B.; Shashi, B. *New Phytol.* **2005**, *165*, 9–28.
5. Espin, J. C.; Garcia-Conesa, M. T.; Tomas-Barberian, F. A. *Phytochem.* **2007**, *68*, 2986–3008.
6. Jassim, S. A.; Naji, M. A. *J. Appl. Microbiol.* **2003**, *93*, 412–427.
7. Iriti, M.; Faoro, F. *Nat. Prod. Commun.* **2009**, *4*, 611–634.
8. Howell, A. B. *Mol. Nutr. Food Res.* **2007**, *51*, 732–737.
9. Kontiokari, T.; Nutinen, M.; Uhari, M. *Pediatr. Nephrol.* **2004**, *19*, 378–383.
10. Vetten, D. A.; Ghaedian, R.; Shetty, K. *Asia Pac. J. Clin. Nutr.* **2005**, *14*, 120–130.
11. Chung, K.-T.; Wong, T. Y.; Wei, C. I.; Huang, Y.-W.; Lin, Y. *Crit. Rev. Food Sci. Nutr.* **1998**, *38*, 421–464.
12. Docherty, J. J.; Fu, M. M.; Hah, J. M.; Faith, S. A.; Booth, T. *Antiviral Res.* **2005**, *67*, 155–162.
13. Khan, H.; Ather, A. *Biotechnol. Annu. Rev.* **2007**, *13C*, 223–264.
14. Weiss, E. I.; Lev-Dor, R.; Kashaman, Y.; Goldhar, J.; Sharon, N.; Ofek, I. *J. Am. Dent. Assoc.* **1998**, *129*, 1719–1723.
15. Weiss, E. I.; Houri-Haddad, Y.; Greenbaum, N.; Hochman, N.; Ofek, I.; Zakay-Rones, Z. *Antiviral Res.* **2005**, *66*, 9–12.
16. Jin, Y.; Cheng, W. X.; Yang, X. M.; Jin, M.; Zhang, Q,; Xu, Z. Q.; Yu, J. M.; Zhu, L.; Yang, S. H.; Liu, I. N.; Cui, S. X.; Fang, Z. Y.; Duan, Z. J. *J. Clin. Virol.* **2009**, *44*, 238–241.
17. Wilhelmi de Cal, I.; Mohedano del Pozo, R. B.; Sánchez-Fauquier, A. *Enferm. Infecc. Microbiol. Clin.* **2008**, *13S*, 61–65.
18. Bodet, C.; Grenier, D.; Chandad, F.; Ofek, I.; Steinberg, D.; Weiss, I. E. *J. Agric. Food Chem.* **2009**, *56*, 627–629.
19. Cos, P.; De Bruyne, T.; Hermans, N.; Apers, S.; Berghe, D. V.; Vlietinck, A. *J. Cur. Med. Chem.* **2003**, *10*, 1345–1359.

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20. Lipson, S. M. *Flavor and Health Benefits of Small Fruits*. Agricultural and Food Chemistry Division, Section D, No. 120, Tech. 9, Abstract, 236th American Chemical Society National Meeting & Exposition, Philadelphia, PA, 2008.
21. Lipson, S. M.; Sethi, L.; Cohen, P.; Gordon, R. E; Tan, I. P.; Burdowski, A.; Stotzky, G. *Phytomedicine* **2007a**, *14*, 23–30.
22. Lipson, S. M.; Cohen, P.; Zhou, J.; Burdowski, A.; Stotzky, G. *Mol. Nutr. Food Res.* **2007b**, *51*, 752–758.
23. Lipson S. M. In *Clinical Microbiology Procedures Handbook*; Isenberg, H. D., Ed.; American Society for Microbiology: Washington, DC, 1992; Vol. 2, pp 8.16.1–8.16.5.
24. Kawai, A.; Fujita, K. *Microbiol. Immunol.* **2007**, *51*, 1071–1079.
25. Patterson, M. K., Jr. *Methods in Enzymology*; Jakoby W. B., Paston, I. H., Eds.; Academic Press: New York, 1979; LVIII, pp 141−152.
26. Hübner, W.; McNerney, G. P.; Chen, P.; Dale, B. M; Gordon, R. E.; Chuang, F. Y.; Li, X. D.; Asmuth, D. M.; Huser, T.; Chen, B. K. *Science* **2009**, *323*, 1743–1747.
27. Lipson, S. M.; Poshni, I. A.; Ashley, R. L.; Grady, L. J.; Ciamician, Z.; Teichberg, S. *FEM Microbiol.* **1993**, *113*, 175–182.
28. Lipson, S. M.; Kaplan, M. H. *J. Diarr. Dis. Res.* **1992**, *10*, 97–100.
29. Gouvea, V.; Glass, R. I.; Woods, P.; Taniguichi, K.; Clark, H. F.; Forrester, B.; Fang, Z. Y. *J. Clin. Microbiol.* **1990**, *28*, 276–282.
30. Klatt, E. C. Histotechniques, 2008. The Internet Pathology Laboratory for Medical Education. http://library.med.utah.edu/WebPath/HISTHTML/HISTOTCH/HISTOTCH.html.
31. Blanchard, H.; Yu, X.; Coulson, B. S.; von Itzstein, M. *J. Mol. Biol.* **2007**, *36* (1), 1215–1226.
32. Gaglielmi, K. M.; Johnson, E. M.; Stehle, T.; Dermody, T. S. *Curr. Top. Microbiol. Immunol.* **2006**, *309*, 1–38.
33. Lopez, S.; Arias, C. F. *Microbiol. Immunol.* **2006**, *309*, 39–66.
34. Krenin, L.; Steitz, M.; Schlicht, C.; Kurth, H.; Gaedcke, F. *Pharmazie* **2007**, *62*, 803–812.
35. Mullen, W.; Marks, S. C.; Crozier, A. *J. Agric. Food Chem.* **2007**, *55* (35), 3148–3157.
36. Mayer, R.; Stecher, G.; Wuerzner, R.; Silva, R. C.; Sultana, T.; Trojier, L.; Feuerstein, I.; Kreig, C.; Abel, G.; Popp, M.; Bobleter, M.; Bonn, G. K. *J. Agric. Food Chem.* **2008**, *56*, 6959–696.
37. Foo, L. Y.; Lu, Y.; Howell, A. B.; Vorsa, N. *J. Nat. Prod.* **2000**, *63*, 1225–1228.
38. Howell, A. B.; Vorsa, N.; der Mardorosian, A.; Foo, L. Y. *N. Eng. J. Med.* **1998**, *339*, 1085–1086.
39. Howell, A. B.; Reed, J. D.; Krueger, C. G.; Winterbottom, R.; Cunningham, D. G.; Leahy, M *Phytochemistry* **2005**, *66*, 2281–2291.
40. Prior, R. L.; Gu, L. *Phytochemistry* **2005**, *66*, 2264–2280.
41. Konowalchuk, J.; Spears, J.I. *Appl. Environ. Microbiol.* **1976**, *32*, 757–763.
42. Konowalchuk, J.; Spears, J. I. *Appl. Environ. Microbiol.* **1978**, *35*, 1219−1220.

Downloaded by CORNELL UNIV on June 26, 2012 | http://pubs.acs.org
Publication Date (Web): March 18, 2010 | doi: 10.1021/bk-2010-1035.ch011

43. Lipson, S. M.; Ashraf, A. B.; Lee, S. H.; Kaplan, M. H.; Shepp, D. H. *J. Clin. Microbiol.* **1995**, *33*, 1411–1413.
44. Karthikeyan, L.; Maurel, P.; Rauch, U.; Margolis, R. K.; Margolis, R. U. *Biochem. Biophys. Res. Commun.* **1992**, *188*, 395–401.
45. Tempaku, A. *J. Health Sci.* **2004**, *51*, 237−241.
46. Laemmli, U. K. *Nature* **1970**, *227*, 680–685.
47. Hagerman, A. E.; Butler, L. G. *J. Biol. Chem.* **1981**, *256*, 4494–4497.
48. Hoffman, T.; Glabasnia, A.; Schwarz, B.; Wisman, K. N.; Gangwer, K. A.; Hagerman, B. *J. Agric. Food Chem.* **2006**, *54* (49), 9503–9509.
49. Jöbstl, E.; O'Connell, J.; Patrick, J; Fairclough, A.; Williamson, M. P. *Biomacromolecules* **2004**, *5*, 942–949.
50. Middleton, E., Jr.; Kandaswami, C,; Theoharides, T. C. *Pharmacol. Rev.* **2000**, *52*, 673–751.
51. Nagai, T.; Suzuki, Y.; Tomimori, T.; Yamada, H. *Biol. Pharm. Bull.* **1995**, *18*, 295–299.
52. Nagai, T,; Miyaichi, Y.; Tomimoni, T.; Suzuki, Y.; Yamada, H. *Antiviral. Res.* **1992**, *19*, 207–217.

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Chapter 12

Flavonoid Fractions from Cranberry Extract Inhibit Growth of Human Tumor Cell Lines

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In spite of improvements in cancer chemotherapy, approximately 50% of cancers are not cured and improvement is needed. Given the association of diets high in fruits and vegetables with lower cancer risk, these foodstuffs have been investigated as sources of compounds to both prevent cancer and treat established tumors. Flavonoids, found in high amounts in cranberries, contribute to improved human health. A flavonoid-rich extract of cranberries (Fr6) and isolated polymers of the flavan-3-ol class of cranberry flavonoids (proanthocyanidins, PACs) were investigated for anticancer activity. Both Fr6 and PACs inhibited proliferation of various human cancer cell lines when grown in culture (*in vitro*). When Fr6 or PACs were administered by injection to mice bearing explants of human tumor cell lines, growth of the tumors was delayed. When treating the prostate tumor cell line DU145 explant, 50% of tumors completely regressed. Both Fr6 and PACs were able to block cell cycle progression and induce cell death. Subfractionation of Fr6 by high performance liquid chromatography yielded fractions containing 3 detectable

In Flavor and Health Benefits of Small Fruits; Qian, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

peaks that exhibited most of the *in vitro* anticancer activity. Work continues on identifying these compounds, which appear to differ chemically from those identified in other laboratories. It is proposed that several components of cranberry will be identified for use in humans as a cancer preventive dietary supplement and, potentially, as a treatment for established tumors.

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Introduction

Major progress has been made in understanding the biology of human cancers, with concomitant design and discovery of novel chemotherapy agents. This has yielded a twofold increase in the number of approved anticancer drugs over the past 10 years. In spite of these gains, there remains a need for agents that can significantly improve life expectancy, either by eradicating tumors completely or by maintaining tumors in a dormant, harmless state. Currently, improvement in patient survival achieved using second and third line treatments is measured in only months or even weeks, at a cost of toxicity to non-cancerous tissues.With a very few important exceptions, cancer cures remain as elusive as they were 10 years ago. Therefore, the quest continues for agents that can inhibit tumor growth and allow patients a normal life expectancy. Treatment with agents that demonstrate selective toxicity to cancer cells while sparing normal cells, or when used concomitantly could enhance the selectivity of commonly used anticancer agents, would be ideal. To this end, many investigators have attempted to isolate components of edible plants as possible agents to selectively control or eradicate cancer without the toxic sequelae (*1–7*). Cranberries (*Vaccinia macrocarpa*) are a rich source of phytochemicals with known health benefits (*8–10*) . Extracts of this berry display antiproliferative activity against human tumor cell lines both *in vitro* and *in vivo* (*8*, *11–20*).

In a preliminary study conducted as collaboration between Ocean Spray, Inc. (Lakeville-Middleboro, MA), and KGK Synergize, Inc. (London, Ontario, Canada), it was observed that ingestion of cranberry juice or freeze-dried presscake slowed the growth and metastatic spread of human tumor cell line explants in immunodeficient (nude) mice (*21*). Since that time, our group has further characterized this *in vivo* inhibition of tumor growth and investigated mechanisms involved in the inhibition of proliferation and induction of cell death by cranberry extracts in human tumor cell lines. Collectively, our results and those of other research groups suggest that cranberries contain constituents that can inhibit tumor growth *in vivo*, to the point of inducing complete eradication of some tumors.

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Materials and Methods

Preparation of Cranberry Extracts Fr6 and PACs

Cranberry extract and fractions were generously provided by Ocean Spray, Inc. Fraction 6 (Fr6) was isolated by standard chromatographic techniques from cranberry presscake as described previously (13). (Presscake is the remaining wet hulls after the juice is squeezed out.) Briefly, a 25EC-water extract of blended cranberry presscake was eluted through a reverse-phase C18 Flash 40M cartridge column (Biotage, Inc., Charlottesville, VA). After elution of inactive material using methanol and water, the Fr6 fraction was eluted using 1% acetic acid/methanol and freeze-dried. The proanthocyanidins (PAC) fraction was isolated from a whole cranberry extract by a similar process, except that the flavonoid-rich fraction (equivalent of Fr6) was subsequently eluted through an LH20 column using 50% ethanol followed by 80% acetone. The concentrations used hereafter refer to those of the freeze-dried powder on a wt:v basis, after solubilizing in the appropriate solvent (DMSO for cell culture, 10% DMSO in water for mouse injections).

Reverse Phase Chromatography of Fr6

As a first step in attempting to isolate individual antiproliferative components from Fr6, a sample of Fr6 (100 µg in 20 µl of methanol) was subjected to reverse-phase chromatography on a C18 column eluted over 20 min with a gradient of 10% to 90% methanol in 0.04% triethylamine/formic acid-buffered water (pH 3.0). Fractions of 4 ml (2 min) were collected, lyophilized, reconstituted in 10% DMSO, and tested for antiproliferative activity as described below. The final concentration of individual fractions tested on the cells was equivalent to a 60 µg/ml treatment of total Fr6.

In Vitro Assays of Antiproliferative Activity

Cell culture medium and fetal bovine serum were purchased from Invitrogen, Inc. (Burlington, Ontario, Canada). Cell culture plasticware was obtained from Invitrogen, Fisher Scientific (Unionville, Ontario), and VWR International (Mississauga, Ontario). The MDA-MB-435 cell line was generously provided by Dr. Janet Price, University of Texas M. D. Anderson Cancer Center (Houston, TX). All other cell lines were purchased from American Type Culture Collection (Rockville, MD). Cultured cell lines were maintained and cytotoxicity assays conducted as described previously (*13*). Briefly, proliferation was assayed in 96-well plates by staining of cells using the vital stain alamarBlue™ (BioSource International, Inc., Camarillo, CA), and quantitated by fluorescence on a Wallac Victor plate reader (Perkin Elmer, Boston, MA). Concentrations of extract that inhibited proliferation of cultured cells by 50% (IC_{50} values) were determined by interpolation of plotted data.

In Vivo Experiments

Female immunocompromised NCR-nu/nu mice, 4-6 weeks old (Taconic, Germantown, NY), were housed in a temperature- and humidity-controlled environment, with a 12 h light:dark cycle, in a pathogen-free isolation facility regulated and controlled by the University of Western Ontario. The mice were maintained according to the "Guide for the Care and Use of Laboratory Animals," set by the Canadian Council on Animal Care and adopted by The University of Western Ontario. Mice were fed a standard laboratory diet *ad libitum*.

Tumors were established by harvesting rapidly proliferating, cultured cells, and injecting them subcutaneously into the right flank. Numbers of cells injected, in 75 :l saline, for each cell line were: U87, 1 x 10^6; HT-29, 5 x 10^6; DU145, 4 x 10^6. When primary tumors become palpable, their maximum length and width were measured every 2 days with calipers. Volume was calculated assuming an ellipsoidal tumor shape (length x width^2 x B/6). When tumors became larger than 1 cm^3, mice were euthanized by CO_2 asphyxiation.

Control groups (8 mice) and groups that received cranberry extracts (6 mice) were administered vehicle (80 :l of 10% DMSO in water), PAC (100 mg/kg), or Fr6 (250 mg/kg), respectively, delivered intraperitoneally (i.p.) in a volume of not more than 80 :l of 10% DMSO in water. Animals in control groups did not exhibit any detrimental effects. Injections were on the following days after tumor implant: 3, 5, 7, 10, 12, 14, 17, 19, 21, 24 (total of 10 injections).

Flow Cytometric Assay of DNA (Cell Cycle) Distribution

Fr6- or PAC-treatment of U87 cells in 75-cm^2 flasks was initiated by introduction of 0.2 volume of a sixfold concentration of Fr6 or PAC in growth medium. Following the indicated incubation period, cells were harvested by trypsinization, and nuclei were prepared, treated with RNase A, and stained using propidium iodide (PI) (*22*). The nuclei were analyzed by flow cytometry (XL-MCL Flow Cytometer, Beckman Coulter, Hialeah, FL). DNA (cell cycle) distributions were analyzed using Multicycle for Windows Advanced DNA Cell Cycle Analysis software (Phoenix Flow Systems, San Diego, CA).

Flow Cytometric Assay of Apoptosis

Cultures were prepared and exposed to Fr6 or PAC as described above. Cells were harvested and, without fixing, stained with fluorescein-conjugated annexin-V (BD Biosciences, Mississauga, ON, Canada) and PI. Cells positive for annexin V and/or PI were considered to be dead or dying.

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Table I. Antiproliferative activity of Fr6 and PAC against a panel of human tumor cell lines (Adapted with permission from references (*13*) and (*14*). Copyrights 2004 American Society for Nutrition and 2006 Lawrence Erlbaum Associates, Inc., respectively.)

		IC_{50} (:g/ml)	
Cell Line	*Tissue*	*Fr6*	*PAC*
U87	glioma	128 ± 25 (7)[a]	47.8 ± 12.2 (7)
HT-29	colon	168 ± 69 (4)	79 ± 5 (2)
DU145	prostate (AR+)[b]	234 ± 75 (4)	95.8 ± 20 (5)
LNCaP	prostate (AR-)	9.9 ± 4.2 (3)	
MCF-7	breast (ER+)	147 ± 22 (3)	
MDA-MB-435	breast (ER-)	212 ± 50. (4)	
SK-MEL-5	melanoma	147 ± 47 (4)	
DMS114	SCLC	21.1 ± 1.9 (4)	

[a] mean ± SD (n). [b] AR - androgen receptor; ER - estrogen receptor; SCLC - small cell lung carcinoma.

Statistical Analysis

Differences in end-points, between treated and control groups, were compared using an unpaired, two-tailed, Student's *t*-test for groups with unequal variances (Microsoft Excel). The end-points were: mean tumor sizes; length of time to reach specified tumor volumes; cell cycle distribution; size of populations of dead and dying cells; relative proliferation of extract-treated cells. In all analyses, differences were considered to be statistically significant if the *P* value was less than 0.05.

Results

A series of human tumor cell lines was screened for sensitivity to cranberry preparations Fr6 and PAC. The cell lines reported in Table I are representative of tumors from 6 different tissues. Sensitivity to cranberry extracts was similar among cell lines, with the exception of the high sensitivity of LNCaP and DMS114 lines to Fr6.

Before testing Fr6 and PAC against tumors in nude mice, it was necessary to determine safe doses at which these preparations could be used in animals. Doses of 100 mg/kg PAC and 250 mg/kg Fr6, injected i.p. every 2 days for 3 weeks, caused minor toxicities that were manageable and from which the mice recovered: skin petechiae that resolved spontaneously after 3 or 4 days, and weight loss (less than 20%) from which the animals recovered during or immediately after the treatment period.

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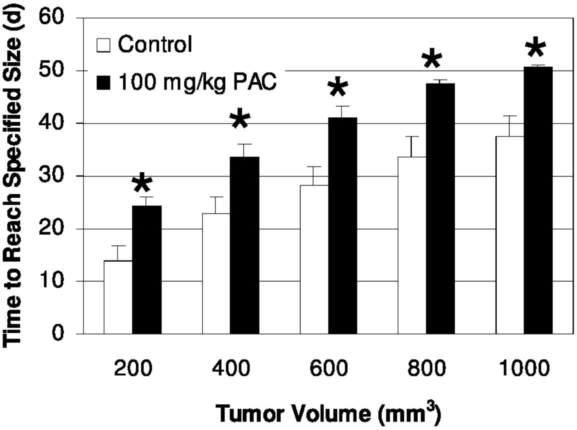

*Figure 1. Effect of PAC treatment on time required for HT-29 tumors to reach milestone sizes in mice. HT-29 colon carcinoma cells (5 x 10^6) were implanted subcutaneously into the flank of female nude mice, and mice received 10 treatments with PAC (control n = 8, treated n = 6; means ± SEM). The length of time for tumors to reach the volumes indicated were determined by interpolation of plotted sizes of tumors versus time, for each individual animal. *: $P < 0.05$. (Reproduced with permission from reference (14). Copyright 2006 Lawrence Erlbaum Associates, Inc.)*

The ability of PAC to inhibit tumor growth *in vivo* was then investigated using tumor cell lines derived from three different tissues: colon, prostate and brain. For these experiments, mice were given 10 injections over 3 weeks, beginning 3 days following inoculation of tumor cells. PAC significantly inhibited the growth of both HT-29 colon tumor and DU145 prostate tumor in nude mice (Figures 1 and 2). PAC significantly delayed HT-29 tumor growth by approximately 10 to 14 d, equivalent to a delay of 75% (the time to reach a volume of 200 mm^3) to 34% (time to reach 1000 mm^3) (Fig. 1). The average size of DU145 prostate tumors at 73 d was decreased by treatment (Figure 2). All control tumors reached a size of 1000 mm^3 (at which point the animals must be euthanized) by 52 to 108 d. None of the control tumors regressed in size prior to reaching 1000 mm^3. However, of the group of 4 PAC-treated tumors, only one reached a size of 600 mm^3. Two of the PAC-treated tumors reached sizes of 400 and 500 mm^3, respectively, but completely regressed by 108 d. A third PAC-treated tumor (585 mm^3 at maximum size) had shrunk to 285 mm^3 when the experiment was ended at 120 d, 96 days after treatment had ended.

PAC treatment of U87 glioma explant-bearing mice increased the time required for the U87 tumors to reach milestone sizes of 400, 500 and 600 mm^3 by approximately 48-59% (5 to 7.5 days; $P < 0.05$; data not shown). This cell line was selected for further characterization of effect of Fr6 and PACs on tumor cell proliferation.

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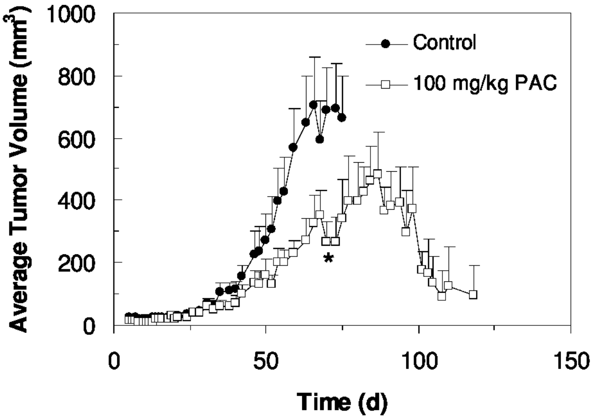

*Figure 2. Effect of PAC treatment on growth of DU145 tumors in mice. DU145 prostate carcinoma cells (4 x 10^6) were implanted subcutaneously into the flank of female, nude mice, and mice received 10 treatments with PAC. (control n = 8, treated n = 6; means ± SEM) *: $P < 0.05$. (Reproduced with permission from reference (14). Copyright 2006 Lawrence Erlbaum Associates, Inc.)*

Fr6 and PAC were tested for their ability to alter cell cycle kinetics of U87 cells by analyzing DNA distribution by flow cytometry after 24 h of exposure (Figure 3). The tested concentrations of Fr6 and PAC inhibited proliferation (i.e., decreased cell numbers at 4 d compared to untreated control cells). Both Fr6 and PAC induced a significant decrease in the proportion of cells in S-phase by 24 h, an increase in G1 fraction, and no change in the G2/M fraction.

The ability of Fr6 and PAC to induce cell death was tested using a flow cytometric, double-staining method for cells actively undergoing apoptosis and dead cells. Cells that were positive for annexin V and PI were considered dying or dead, including those killed by non-apoptotic mechanisms, and are collectively referred to as “dead and dying cells” (Figure 4). At concentrations that caused inhibition of proliferation by 4 d, both cranberry extract preparations induced significant cell death. Paclitaxel was included as a positive control.

Even though Fr6 comprises only 5% of the ingredients in the cranberry presscake extract, it appears that most of Fr6 consists of components that do not have cytotoxic activity. Figure 5 is a chromatogram developed during the fractionation of Fr6. Each fraction was tested for cytotoxic activity against the sensitive LNCaP prostate cancer cell line. Two fractions that contained less than 10% of the material in Fr6 were found to contain the majority of inhibitory activity. Furthermore, a preliminary chromatographic subfractionation of the strongest fraction from this elution indicates that a minor component of this fraction may contain most of the inhibitory activity (Freeman and Ferguson, *data not shown*).

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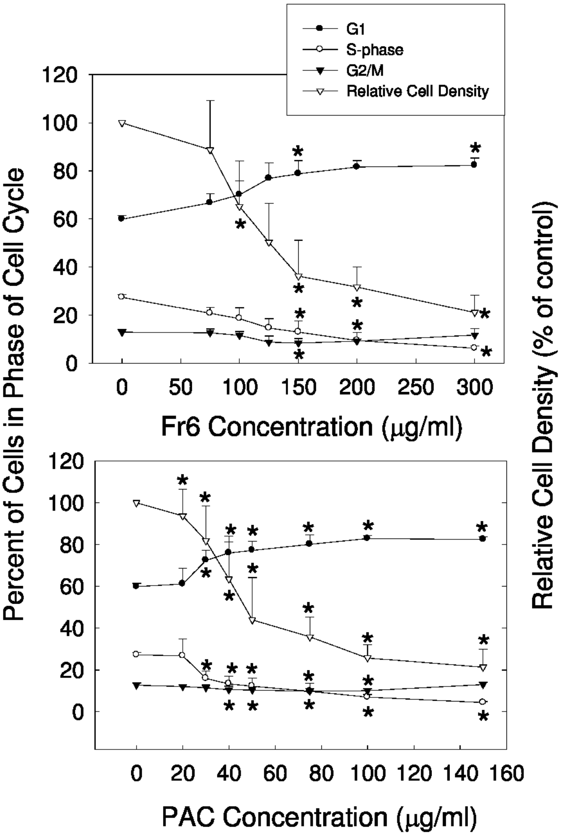

*Figure 3. Cell cycle distribution analysis of Fr6-treated and PAC-treated U87 cells. Data points represent summarized data from 3 to 5 separate determinations (means ± SD). The percentages of cells in G0/G1 phase, S phase and G2/M phases were determined at 24 h. Relative cell density (96-well plate, alamar Blue assay) was determined at 4 d for each exposure for each experiment. IC_{50} values for Fr6 and PAC for this set of experiments were, respectively, 128.4 ± 25.1 mg/L and 47.8 ± 12.2 mg/L (means ± SD, n = 5). *: $P < 0.05$ compared to DMSO control. (Adapted with permission from reference (14). Copyright 2006 Lawrence Erlbaum Associates, Inc.)*

Discussion

The presence of compounds with anticancer activity in safely consumed foods conservatively suggests that agents might be isolated that selectively kill cancer cells, sparing normal tissues and achieving local tumor control or cures. Crude extracts and individual components obtained from fruits and berries are able to not only slow cell growth, but can kill cancer cells outright. However, these activities are observed at concentrations that are not likely achievable through normal dietary consumption. The observed capacity of components to induce cell death suggests that anticancer activity may entail, at least at higher doses, some adverse effect

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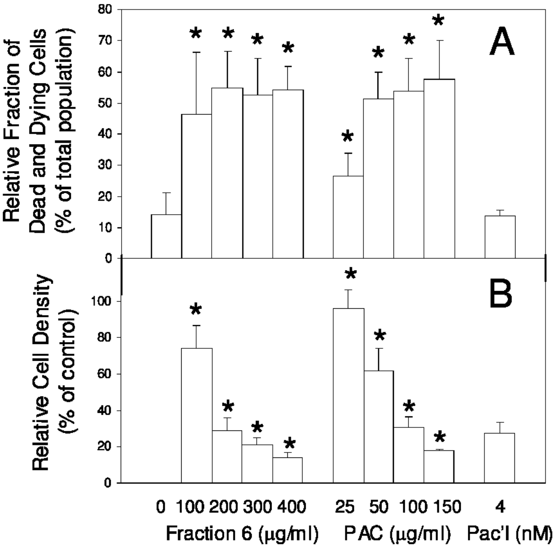

*Figure 4. Induction of cell death in Fr6- and PAC-treated U87 cells. Panel A: after 48 h, cells were tested by staining with annexin V and propidium iodide (Panel A). Bars indicate the percent of the entire population of cells that was dead and dying, summarized from 3 to 4 experiments (means ± SD). Panel B: relative proliferation of treated cells, as a function of live cell density after 4 d (n = 3, means ± SD). Pac'l, paclitaxel. *: P < 0.05 compared to DMSO control. (Adapted with permission from reference (14). Copyright 2006 Lawrence Erlbaum Associates, Inc.)*

on normal cells. Nevertheless, the optimism about anticancer phytochemicals has been fortified by demonstration that crude extracts and purified components inhibit tumor growth in tumor-bearing mice without untoward toxicity to the animal. Therefore, efforts are increasing to purify, identify and characterize individual components of many different fruits and berries with the goal of obtaining agents that prevent cancer formation or progression, or kill tumor cells outright.

Several groups of investigators have made significant contributions to our understanding of the anticancer activity of cranberries. This literature has been reviewed elegantly and thoroughly by Dr. Neto (*9*, *23*, *24*), and so will not be discussed comprehensively in this report. However, it is worthwhile to place these findings in an historical perspective. Following the initial observation by Guthrie that cranberry products could inhibit growth of a human tumor cell line explant in nude mice (*3*, *21*), various groups demonstrated growth inhibition and induction of cell death in tumor cell lines *in vitro* (*11–14*, *16–20*, *25–27*).

Cranberry extracts also inhibit cellular activities associated with malignant progression and tumor aggressiveness (*15*). Most of these studies utilized extracts that were enriched in flavonoids and/or PACs, but it was not known which components were responsible for the cytotoxic activity. The individual components have now been well characterized, and some are strong candidates as mediators of anticancer activity. Cranberries contain compounds from each of

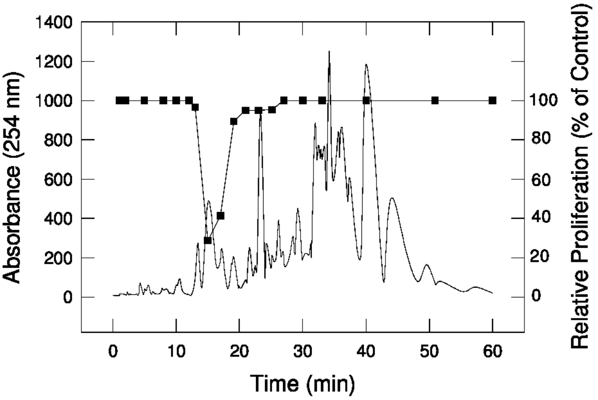

Figure 5. Inhibition of proliferation of LNCaP prostate tumor cell line by subfractions of Fr6, separated by HPLC. Fractions of 4 ml were lyophilized, reconstituted in 10% DMSO, and assayed for the ability to inhibit proliferation of LNCaP cells (closed boxes). The graph of the HPLC elution represents ultraviolet absorbance of the various peaks that were eluted.

four major groups of flavonoids: proanthocyanidins, anthocyanins, flavan-3-ols (catechins) and flavonols (*28–32*). Glycosides of quercetin and myricetin qualify as potential candidate anticancer compounds from cranberry (*8*, *24*, *33–37*), but have not been unequivocally demonstrated as such in purified cranberry extracts. Some purified components have, in fact, been demonstrated to inhibit tumor cell growth, including hydroxycinnamoyl ursolic acid (*11*) and a family of PACs (related by their subunit structure and ranging in size from 2 to 12 units in length) (*12*, *27*). The definitive experiment will be to demonstrate antitumor activity of these compounds *in vivo*.

The extracts used in the studies reported herein were obtained from cranberry presscake (the material remaining following the extraction of juice) (Fr6) or from homogenized whole berry (PAC). The initial extract from whole cranberry or from presscake contains sugars, proteins, acids, and waxy materials that have no antiproliferative activity. These are separated from Fr6 and PACs by the initial chromatographic process. Both sources are believed to yield the majority of flavonoids in cranberry, but PACs are expected to be exclusive of flavonols and their glycosides. Both Fr6 and PACs significantly inhibited proliferation of tumor cell lines *in vitro* (*13*, *14*). PACs inhibited the growth of 3 cell lines *in vivo*, and Fr6 inhibited growth of U87 *in vivo*.

The effect of the PAC fraction in inducing regression of explant tumors of the androgen receptor-negative cell line DU145 raises the possiblity of a clinical role for this family of polymeric epicatechins. Prostate tumors are generally refractory to chemotherapy after they have converted from being anti-androgen responsive to non-responsive (*38*), indicating a major need for alternate therapy. Plant and fruit flavonoids inhibit proliferation of the DU145 cell line (*30*, *39*). Therefore some prostate tumors may be sensitive to these components.

The mechanisms by which flavonoids inhibit tumor growth are only partially understood. Flavonoids can induce cell death or cell cycle arrest depending on the

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flavonoid and on the concentration (*5*, *40–44*). Also, arrest may occur in G1 or G2/M, depending on the cell type (*5*, *13*, *14*, *40*). This variation between G1 and G2/M arrest was observed between our cell lines using the same preparation of Fr6 (*13*, *14*). Optimal use of flavonoids to control cancer will require significant progress in mechanistic studies.

Besides our demonstration of antitumor activity *in vivo*, there has been only one other report of *in vivo* inhibition of tumor explant growth (*19*). The observed *in vivo* anticancer activity of cranberry extract, with minimal toxicity to the host animal, suggests that certain components of cranberries may have selective inhibitory activity against tumor cells and therefore may have clinical applicability as a treatment against established tumors. Cranberry also demonstrates cancer preventive activity (*23*, *45*), a property that could be an important basis of a preventative dietary supplement. Therefore, cranberry or components thereof are expected to act as cancer preventive agents, not only through their antioxidant properties [which are not discussed above but have been reviewed elsewhere (*8*, *24*, *25*)], but possibly through cytotoxic action against microscopic tumors or premalignant foci. Such small clusters of tumor cells, particularly those in a proliferative state, may be more sensitive to low concentrations of circulating cranberry constituents achieved by dietary consumption. More needs to be understood, however, about the pharmacokinetics of active components, what concentrations are achievable when consumed safely and comfortably in the diet, and whether the isolated active components will function as efficiently as a mixture. Clearly, there remain several important questions to be answered by future studies in order to optimize benefit from this valuable berry.

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Abbreviations

AR, androgen receptor; ER, estrogen receptor; Fr6, Fraction 6 of cranberry presscake extract; IC_{50}, concentration that inhibits proliferation by 50%; i.p., intraperitoneally; PAC, proanthocyanidin fraction from whole cranberry; PI, propidium iodide.

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References

1. Waladkhani, A. R.; Clemens, M. R. Effect of dietary phytochemicals on cancer development. *Int. J. Mol. Med.* **1998**, *1*, 747–753.
2. Hollman, P. C.; Katan, M. B. Health effects and bioavailability of dietary flavonoids. *Free Radical. Res.* **1999**, *31* (Suppl.), S75–S80.
3. Guthrie, N.; Carroll, K. K. Inhibition of Human Breast Cancer Cell Growth and Metastasis in Nude Mice by Citrus Juices and Their Constituent Flavonoids. In *Biological Oxidants and Antioxidants. Molecular Mechanisms and Health Effects*; Packer, L., Ong, A.S.H., Eds.; AOCS Press: Champaign, IL, 1998; pp 310−316.
4. Brownson, D. M.; Azios, N. G.; Fuqua, B. K.; Dharmawardhane, S. F.; Mabry, T. J. Flavonoid effects relevant to cancer. *J. Nutr.* **2002**, *132*, 3482S–3489S.
5. Choi, J. A.; Kim, J. Y.; Lee, J. Y.; Kang, C. M.; Kwon, H. J.; Yoo, Y. D.; Kim, T. W.; Lee, Y. S.; Lee, S. J. Induction of cell cycle arrest and apoptosis in human breast cancer cells by quercetin. *Int. J. Oncol.* **2001**, *19*, 837–844.
6. Liao, S.; Umekita, Y.; Guo, J.; Kokontis, J. M.; Hiipakka, R. A. Growth inhibition and regression of human prostate and breast tumors in athymic mice by tea epigallocatechin gallate. *Cancer Lett.* **1995**, *96*, 239–243.
7. Chen, Z. P.; Schell, J. B.; Ho, C. T.; Chen, K. Y. Green tea epigallocatechin gallate shows a pronounced growth inhibitory effect on cancerous cells but not on their normal counterparts. *Cancer Lett.* **1998**, *129*, 173–179.
8. He, X.; Liu, R. H. Cranberry phytochemicals: isolation, structure elucidation, and their antiproliferative and antioxidant activities. *J. Agric. Food Chem.* **2006**, *54*, 7069–7074.
9. Neto, C. C. Cranberry and its phytochemicals: a review of in vitro anticancer studies. *J. Nutr.* **2007**, *137*, 186S–193S.
10. Henig, S. Y.; Leahy, M. H. Cranberry juice and urinary-tract health: science supports folklore. *Nutrition* **2000**, *16*, 684–687.
11. Murphy, B. T.; MacKinnon, S. L.; Yan, X.; Hammond, G. B.; Vaisberg, A. J.; Neto, C. C. Identification of triterpene hydroxycinnamates with *in vitro* antitumor activity from whole cranberry fruit (*Vaccinium marcocarpon*). *J. Agric. Food Chem.* **2003**, *51*, 3541–3545.
12. Seeram, N. P.; Adams, L. S.; Hardy, M. L.; Heber, D. Total cranberry extract versus its phytochemical constituents: Antiproliferative and synergistic effects against human tumor cell lines. *J. Agric. Food Chem.* **2004**, *52*, 2512–2517.
13. Ferguson, P. J.; Kurowska, E.; Freeman, D. J.; Chambers, A. F.; Koropatnick, D. J. A flavonoid fraction from cranberry extract inhibits proliferation of human tumor cell lines. *J. Nutr.* **2004**, *134*, 1529–1535.
14. Ferguson, P. J.; Kurowska, E.; Freeman, D. J.; Chambers, A. F.; Koropatnick, D. J. *In vivo* inhibition of growth of human tumor lines by flavonoid fractions from cranberry extract. *Nutr. Cancer* **2006**, *56*, 86–94.
15. Kondo, M.; Lamoureaux, T. L.; Neto, C. C.; Hurta, R. A. R.; Curtis, S.; Matchett, M. D.; Yeung, H.; Sweeney-Nixon, M. I.; Vaisberg, A. J. Proanthocyanidins, anthocyanins, and triterpenoids from cranberry fruits:

Downloaded by PENNSYLVANIA STATE UNIV on June 25, 2012 | http://pubs.acs.org
Publication Date (Web): March 18, 2010 | doi: 10.1021/bk-2010-1035.ch012

Antitumor activity and effects on matrix metalloproteinase expression. *J. Nutr.* **2004**, *134* (12S), 3538S.

16. Seeram, N. P.; Adams, L. S.; Zhang, Y.; Lee, R.; Sand, D.; Scheuller, H. S.; Heber, D. Blackberry, black raspberry, blueberry, cranberry, red raspberry, and strawberry extracts inhibit growth and stimulate apoptosis of human cancer cells in vitro. *J. Agric. Food Chem.* **2006**, *54*, 9329–9333.
17. Boivin, D.; Blanchette, M.; Barrette, S.; Moghrabi, A.; Béliveau, R. Inhibition of cancer cell proliferation and suppression of TNF-induced activation of NF6B by edible berry juice. *Anticancer Res.* **2007**, *27*, 937–948.
18. Kresty, L. A.; Howell, A. B.; Baird, M. Cranberry proanthocyanidins induce apoptosis and inhibit acid-induced proliferation of human esophageal adenocarcinoma cells. *J. Agric. Food Chem.* **2008**, *56*, 676–680.
19. Hochman, N.; Houri-Haddad, Y.; Koblinski, J.; Wahl, L.; Roniger, M.; Bar-Sinai, A.; Weiss, E. I.; Hochman, J. Cranberry juice constituents impair lymphoma growth and augment the generation of antilymphoma antibodies in syngeneic mice. *Nutr. Cancer* **2008**, *60*, 511–517.
20. Liu, M.; Lin, L. Q.; Song, B. B.; Wang, L. F.; Zhang, C. P.; Zhao, J. L.; Liu, J. R. Cranberry phytochemical extract inhibits SGC-7901 cell growth and human tumor xenografts in Balb/c nu/nu mice. *J. Agric. Food Chem.* **2009**, *57*, 762–768.
21. Guthrie, N. Effect of cranberry juice and products on human breast cancer cell growth. *FASEB J.* **2000**, *14*, 531.13.
22. Vindeløv, L. L.; Christensen, I. J.; Nissen, N. I. A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. *Cytometry* **1983**, *3*, 323–327.
23. Neto, C. C. Cranberry and blueberry: evidence for protective effects against cancer and vascular diseases. *Mol. Nutr. Food Res.* **2007**, *51*, 652–664.
24. Neto, C. C.; Amoroso, J. W.; Liberty, A. M. Anticancer activities of cranberry phytochemicals: an update. *Mol. Nutr. Food Res.* **2008**, *52*, S18–S27.
25. Yan, X.; Murphy, B. T.; Hammond, G. B.; Vinson, J. A.; Neto, C. C. Antioxidant activities and antitumor screening of extracts from cranberry fruit (Vaccinium macrocarpon). *J. Agric. Food Chem.* **2002**, *50*, 5844–5849.
26. Sun, J.; Liu, R. H. Cranberry phytochemical extracts induce cell cycle arrest and apoptosis in human MCF-7 breast cancer cells. *Cancer Lett.* **2006**, *241*, 124–134.
27. Singh, A. P.; Singh, R. K.; Kim, K. K.; Satyan, K. S.; Nussbaum, R.; Torres, M.; Brard, L.; Vorsa, N. Cranberry proanthocyanidins are cytotoxic to human cancer cells and sensitize platinum-resistant ovarian cancer cells to paraplatin. *Phytother. Res.* **2009**, *23*, 1066–1074.
28. Foo, L. Y.; Lu, Y.; Howell, A. B.; Vorsa, N. A-type proanthocyanidin trimers from cranberry that inhibit adherence of uropathogenic P-fimbriated *Escherichia coli*. *J. Nat. Prod.* **2000**, *63*, 1225–1228.
29. Hammerstone, J. F.; Lazarus, S. A.; Schmitz, H. H. Procyanidin content and variation in some commonly consumed foods. *J. Nutr.* **2000**, *130* (8S Suppl), 2086S–2092S.

30. Kampa, M.; Hatzoglou, A.; Notas, G.; Damianaki, A.; Bakogeorgou, E.; Gemetzi, C.; Kouroumalis, E.; Martin, P. M.; Castanas, E. Wine antioxidant polyphenols inhibit the proliferation of human prostate cancer cell lines. *Nutr. Cancer* **2000**, *37*, 223–233.
31. Kandil, F. E.; Smith, M. A.; Rogers, R. B.; Pepin, M. F.; Song, L. L.; Pezzuto, J. M.; Seigler, D. S. Composition of a chemopreventive proanthocyanidin-rich fraction from cranberry fruits responsible for the inhibition of 12-O-tetradecanoyl phorbol-13-acetate (TPA)-induced ornithine decarboxylase (ODC) activity. *J. Agric. Food Chem.* **2002**, *50*, 1063–1069.
32. Gu, L.; Kelm, M.; Hammerstone, J. F.; Beecher, G.; Cunningham, D.; Vannozzi, S.; Prior, R. L. Fractionation of polymeric procyanidins from lowbush blueberry and quantification of procyanidins in selected foods with an optimized normal-phase HPLC-MS fluorescent detection method. *J. Agric. Food Chem.* **2002**, *50*, 4852–4860.
33. Yang, J. H.; Hsia, T. C.; Kuo, H. M.; Chao, P. D.; Chou, C. C.; Wei, Y. H.; Chung, J. G. Inhibition of lung cancer cell growth by quercetin glucuronides via G2/M arrest and induction of apoptosis. *Drug Metab. Dispos.* **2006**, *34*, 296–304.
34. Lu, J.; Papp, L. V.; Fang, J.; Rodriguez-Nieto, S.; Zhivotovsky, B.; Holmgren, A. Inhibition of mammalian thioredoxin reductase by some flavonoids: Implications for myricetin and quercetin anticancer activity. *Cancer Res.* **2006**, *66*, 4410–4418.
35. De Leo, M.; Braca, A.; Sanogo, R.; Cardile, V.; DeTommasi, N.; Russo, A. Antiproliferative activity of *Pteleopsis suberosa* leaf extract and its flavonoid components in human prostate carcinoma cells. *Planta Med.* **2006**, *72*, 604–610.
36. Kuntz, S.; Wenzel, U.; Daniel, H. Comparative analysis of the effects of flavonoids on proliferation, cytotoxicity, and apoptosis in human colon cancer cell lines. *Eur. J. Nutr.* **1999**, *38*, 133–142.
37. Ichimatsu, D.; Nomura, M.; Nakamura, S.; Moritani, S.; Yokogawa, K.; Kobayashi, S.; Nishioka, T.; Miyamoto, K. Structure-activity relationship of flavonoids for inhibition of epidermal growth factor-induced transformation of JB6 Cl 41 cells. *Mol. Carcinog.* **2007**, *46*, 436–445.
38. Lee, E. C.; Tenniswood, M. P. Emergence of metastatic hormone-refractory disease in prostate cancer after anti-androgen therapy. *J. Cell. Biochem.* **2004**, *91*, 662–670.
39. Tyagi, A.; Agarwal, C.; Agarwal, R. The cancer preventive flavonoid silibinin causes hypophosphorylation of Rb/p107 and Rb2/p130 via modulation of cell cycle regulators in human prostate carcinoma DU145 cells. *Cell Cycle* **2002**, *1*, 137–142.
40. Casagrande, F.; Darbon, J. M. Effects of structurally related flavonoids on cell cycle progression of human melanoma cells: Regulation of cyclin-dependent kinases CDK2 and CDK1. *Biochem. Pharmacol.* **2001**, *61*, 1205–1215.
41. Bhatia, N.; Agarwal, R. Detrimental effect of cancer preventive phytochemicals silymarin, genistein and epigallocatechin 3-gallate on

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Publication Date (Web): March 18, 2010 | doi: 10.1021/bk-2010-1035.ch012

epigenetic events in human prostate carcinoma DU145 cells. *Prostate* **2001**, *46*, 98–107.

42. Gupta, S.; Ahmad, N.; Nieminen, A. L.; Mukhtar, H. Growth inhibition, cell-cycle dysregulation, and induction of apoptosis by green tea constituent (-)-epigallocatechin-3-gallate in androgen-sensitive and androgen-insensitive human prostate carcinoma cells. *Toxicol. Appl. Pharmacol.* **2000**, *164*, 82–90.
43. Park, J. H.; Oh, E. J.; Choi, Y. H.; Kang, C. D.; Kang, H. S.; Kim, D. K.; Kang, K. I.; Yoo, M. A. Synergistic effects of dexamethasone and genistein on the expression of Cdk inhibitor p21WAF1/CIP1 in human hepatocellular and colorectal carcinoma cells. *Int. J. Oncol.* **2001**, *18*, 997–1002.
44. Murai, T.; Nakagawa, Y.; Maeda, H.; Terada, K. Altered regulation of cell cycle machinery involved in interleukin-1-induced G(1) and G(2) phase growth arrest of A375S2 human melanoma cells. *J. Biol. Chem.* **2001**, *276*, 6797–6806.
45. Prasain, J. K.; Jones, K.; Moore, R.; Barnes, S.; Leahy, M.; Roderick, R.; Juliana, M. M.; Grubbs, C. J. Effect of cranberry juice concentrate on chemically-induced urinary bladder cancers. *Oncol. Rep.* **2008**, *19*, 1565–1570.

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Chapter 13

Açai (*Euterpe oleracea*)

An Amazonian Palm Fruit with Broad Antioxidant and Anti-Inflammatory Activities

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The pulp of the Amazonian palm fruit, açai (*Euterpe oleracea* Mart.), has been found to have exceptional antioxidant capacity *in vitro*, particularly against the superoxide, peroxyl, and hydroxyl radicals. Studying foods for their potential health benefits to humans can benefit from *in vitro* assays that can correlate with *in vivo* outcomes. Using the cell-based protection of erthrocytes (CAP-e) assay can determine if red blood cells exposed to foods allow antioxidants to enter into cells and protect cells from oxidative damage. Using this assay, the reduction in fluoresecence has been shown to be proportional to the level of intracellular antioxidant protection. In comparing results from blood samples taken during a randomized, double-blind, placebo-controlled, cross over study involving an açai-based fruit juice taken by subjects in a state of oxidative stress, the CAP-e assay correlated well with levels of antioxidant compounds in cells and reduction in lipid peroxidation. A dose dependent reduction in formation of reactive oxygen species in polymorphonuclear (PMN) cells has shown açai pulp to be active even when diluted down to 0.1 picogram/mL, suggestive of cellular signaling, and indicative that the pulp may contain compounds with the potential to be a

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food able to exhibit potent anti-inflammatory activity *in vivo*. A pilot study on range of motion and joint pain in older adults supports this hypothesis, when studied in in older subjects with mild to moderate inflammation of the knees and lumbar spine.

Introduction

The palm fruit, *Euterpe olearace* Martius (*E. oleracea*), is one of three species of edible palm fruit that are members of the genus *Euterpe*. These three species, *E. olearacea*, *E. edulis*, and *E. precatoria*, are only found growing within the boundaries of the Amazon region, particularly Brazil. Natives living in Brazil refer to these three fruit species by the same name, *açai* (pronounced ah sigh ee), although the fruit of *E. edulis* is also referred to as *jussara* or *jucara*.

The most dominant fruit gathered commercially as a food or nutraceutical ingredient is that of *E. oleracea*. The pulp of this fruit is primarily processed for use as a juice for domestic consumption and export. The fruit is harvested in the Brazilian states of Para and Amapa, where it is found growing in abundance among the hundreds of islands within the complex floodplain ecosytem of the Amazon River and its tributaries.

Due to its perishability, it is rapidly shipped after harvesting to regional processing facilities to be made into frozen pulp, or further processed into freeze-dried or spray-dried powders. These powders can be added to water or fruit/berry concentrates to produce açai-based juices. Generally, açai pulp is mixed with more flavorful fruits and/or berries to improve taste, as the pulp tastes slightly chalky. Fresh fruit is not available outside of the harvest season domestically, nor found sold in its whole fruit form outside of Brazil, due to its perishable nature.

In Belem, the largest city within the Amazon basis, located in Para state, with a population exceeding one million residents, daily per capita consumption of açai pulp juice is estimated to be nearly two liters a day from July to December. Açai fruit stands that expel the seed and provide açai juice are found all over the city, sometimes with several retail açai juice stands no more than a few blocks apart.

A characteristic of the species is its growth in clumps, a result of basal suckering. The number of these clumps varies and can reach 25, including suckers, depending on environmental conditions (*1*). The palm has a thin trunk and averages 15 to 20 meters in height. The number of fruit clusters varies, with three to five common. Each cluster of infloresence-producing fruit is at a different stage of development, allowing for several harvests of fruit from the same palm tree within a six month period. The fruit is round and between 1.5 to 2.5 cm in diameter; the edible mesocarp of the fruit is dark purple.

Like most fruits found in the Amazon, it is highly perishable. Açai palm trees are found abundantly growing over a range of approximately 7 million square kilometers within the eastern portion of the Amazon, with the highest density of palms, up to 7,000 trees per hectare, located in the Brazilian states of Para and Amapa. Locals living in this region harvest the fruit from June through December.

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Harvesting the fruit requires climbing to within a few meters of the top of the palm to remove the mature fruit clusters, which are attached to the infloresence. Each cluster can weigh between one to two kilos. Accomplished harvesters can swing from one palm to another and remove several clusters before returning to the ground, where the fruit is stripped and placed in woven baskets for transport. Transport almost always is done by canoe or boat where baskets are aggregated into the hulls and then transferred into larger vessels that can take up to 2,000 fourteen kilo baskets to collection points where they are transferred to processing facilities, a cycle that goes on seven days a week throughout the dry season.

Processing of the fruit to preserve it nutritional density, organoleptic qualities, and phytochemical composition, must be performed within 18-20 hours of harvesting. Thereafter the fruit pulp will diminish in quality and desirability. When processed, the skin is soaked for removal, leaving behind the pulp and seed. The seed constitutes between 82% to 89% of the fruit, and is expelled, leaving only the pulp to be further processed for various food uses, the most common of which is as a juice. The percentage of solids in the pulp following processing varies between 14% to 6%, with the higher percentage considered the highest grade of pulp.

The nutrient composition of de-seeded açai fruit was first reported in 1945 (*2*). Açai and farinha (*Manihot esculenta*) (derived from cassava – an edible starchy tuberous root), can constitute up to two-thirds of the calories found in the diet of natives that live in thousands of rural villages and communities within the flood plains (*varzea*) where the fruit grows abundantly.

In 2006, the nutritional composition of açai pulp for its vitamins, minerals, amino acids, and fatty acids, and phytochemical composition, particularly of polyphenols, was reported by chemists working in several labs in the United States (*3*). The pulp was found to have a surprisingly high concentration of mono- and poly-unsaturated fatty acids; 82% of its unsaturated fatty acid composition in freeze-dried samples of açai pulp due to the presence of oleic acid and linoleic acid, higher in unsaturated fatty acids than found in olive oil or avocado oil, but likely comparable in concentration given variability of these oils and açai pulp (*3*).

Assessment of Safety Based on Historical Consumption and Toxicology Studies

A study of the diet of paleoindian cave dwellers in the Amazon has confirmed that a significant energy source for humans living during the Pleistocene and Holecene occupations included palm fruit (*4–6*).

The use of açai as a food source in the Amazon was chronicled by British, Portuguese and American explorers beginning with the 18th century account of noted botanist, Joseph Banks, who visited Brazil in 1768. In an entry to his diary dated December 7, 1768, Banks described the Açai fruit as "*palm berries that appear much like black grapes but for eating have scarcely any pulp covering a very large stone*" (*7*). Renowned American naturalist, William H. Edwards, lived in Para state in 1846. In describing the importance of the fruit in the local diet, he wrote: "*From various palm fruits are prepared substances in great request among difference classes of people ... The fruit is covered by a thick skin, beneath which, imbedded in a very slight pulp, is the stone. Warm water is poured on, to loosen the skin, and berries are briskly rolled together in a large vessel. The stones are thrown out, the liquid strained off the skins, and there is left a thick, cream-like substance, of a purple color…To a stranger, the taste is, usually, disagreeable, but soon, it becomes more prized than all fruits beside, and is as much a necessity as one's dinner*" (*8*).

A more recent account of how little has changed since 1846 in terms of local preparation of the fruit as a juice is described by researchers affiliated with the Nature Conservancy who reported in 2004 that, "*The rind (mesocarp) of the açai fruit is ground and mixed with water to form the thick, purple vinho, which forms a staple part of the ribeirinho diet. In towns and cities, açai is processed into vinho at small stands or shops and sold by the litre...*"((*9*), p. 318)

A series of unpublished toxicology studies have been performed of açai pulp either in the form of freeze-dried pulp powder or as found in a juice product whose ingredient is predominantly acai pulp (freeze-dried and frozen) (Monavie Active®, Monavie LLC, Salt Lake City, UT, USA). In an acute oral toxicology study by gavage, administration of single oral doses of 5,000 mg/kg body weight (bw) and 20,000 mgkg bw, observed for lethality and toxic symptoms for 14-days post treatment, resulted in no lethality, clinical symptoms, or gross pathology. Hence, the single oral LD50 is determinated to be higher than 20,000 mg/kg bw for the juice. An acute toxicology study in rats given 2,000 mg/kg bw of freeze-dried açai pulp (OptiAcai®, K2A, Springville, UT, USA) by intubation, with a 14-day post-treatment observation period, resulted in no lethality, adverse effects, or gross pathology.

Toxicity studies were also performed to assess mitogenicity, mutagenicity and clastogenicity of the açai-based juice. Cell cytotoxicity was not observed following 48 hours of incubating mulitplates containing L929 or Balb/3T3 clone A31 cells using the cytotoxicity test model. Negative findings were reported for the following assays of açai pulp and/or the açai-based fruit juice: bacterial reverse mutagenicity assay (AMES test), mammalian chromosomal aberration test in Chinese hamster V79 cells, mouse peripheral blood micronucleus assay, *in vivo* genotoxicity test by bone marrow micronucleus assay in adult mice BALB/c strain, and the mammalian cell gene mutation (L5178Y/TK mouse lymphoma) assay (unpublished). In the latter, no mutagenic effect was observed with and without metabolic activation. In the mammalian chromosomal aberration assay, when tested up to cytotoxic concentrations, both with and without metabolic activation, it did not induce structural chromosome aberrations in Chinese hamster lung cells. Therefore, it was not found to be clastogenic in this sytem. In the *in vivo* bone marrow micronucleus assay neither oral nor intraperitoneal administration showed an ability to induce an increase of micronuclei frequency in polychromatic erthrocytes in the bone marrow of BALB/c mice. The Ames test showed that neither the juice was mutagenic in using either Salmonella (TA98, TA100, TA1535, and TA1537) or *E. coli* {WP2 (uvrA)] strains tested in triplicate. There were no revertants exceeding the background average either

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with or without metabolic activation, nor any dose-related increase over the range tested. The freeze-dried acai pulp similarly failed to show the test item was mutagenic in either of two experiments in the presence or absence of metabolic activation, tested in triplicate, at concentrations up to 5000 micrograms per plate under the conditions employed.

Comparative Antioxidant Activity

Considerable scientific interest in this fruit arose following the 2006 publication by multiple investigators and laboratories that açai had stronger antioxidant capacity compared to other antioxidant-rich fruits, berries and vegetables tested by the U.S. Department of Agriculture's Agricultural Resesarch Services (USDA-ARS), based on the oxygen radical absorbance capacity (ORAC) assay. The ORAC assay had been used extensively by USDA-ARS, to compare various freeze-dried fruits, vegetables and nuts, for their scavenging capacity against the peroxyl free radical *in vitro* (*10–12*).

Initial evidence reported by Pozo-Insfran and colleagues in 2006, found that açai polyphenolics in their glycoside and aglycone forms induced apoptosis of (HL-60) leukemia cells due to caspase-3 activation. This paper stimulated further investigation of the fruit's bioactivity *in vitro* and *in vivo* (*13*).

The author's present findings report on the use of a novel assay to document the bioavailability of antioxidants in açai pulp, both at the cellular level, and in human subjects, after acute or chronic daily consumption.

Antioxidant and Anti-inflammatory Activity of Açai Pulp

Reactive oxygen species (ROS) are produced in aerobic organisms and are widely believed to play a pivotal role in aging and a number of degenerative diseases. Freeze-dried de-seeded açai pulp powder has been shown to have exceptional antioxidant properties against superoxide ($O_2^{\bullet-}$) by the SOD assay, as well as against the peroxyl radical ($RO_2^{\bullet}$) by the $ORAC_{FL}$ assay. Freeze-dried açai pulp had the highest reported superoxide scavenging capacity *in vitro* of any food tested at the time of publication, at 1,614 SOD units/g (14). The ORAC-hydrophilic/lipophilic score of 1026.9 µmole Trolox equivalent (TE)/g represented a scavenging capacity that exceeded any other freeze-dried fruit or vegetable tested by USDA-ARS, with the exception of certain spices, while also showing moderate squenching capacities against both the peroxynitrite radical ($ONOO^-$) and hydroxyl radical ($OH^{\bullet}$) (*14*).

Based on the content of potent antioxidant compounds found in the fruit reported in 2006 (14), açai pulp was tested in biological assays, to evaluate whether the antioxidants played a specific role in biological systems *in vitro*.

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Testing of Pulp in Fresh Human Cells

As a direct extension of the tests of açai pulp for antioxidant capacity, the pulp was tested in the CAP-e bioassay, which specifically measures to what extent antioxidants can enter into and protect living cells (*15*). It was found that antioxidants in açai pulp were able to cross the lipid bilayer cell membrane of fresh human red cells and provide intracellular protection from oxidative damage (*15*) (Figure 1).

An open trial was conducted, followed by a randomized, double-blind, placebo-controlled cross-over study. The primary antioxidants in the açai-based juice tested (Monavie Active®, Monavie LLC, Salt Lake City, UT) were in the form of polyphenols, predominantly flavonoids such as anthocyanins.

In the first open pilot study, consumption of a single acute dose (120 mL) of the açai-rich juice, following an overnight fast to induce oxidative stress, resulted in a significant increase in antioxidant protection *in vivo*, confirmed by testing blood samples before drinking the juice and at 1 and 2 hours following ingestion. The cell-based antioxidant protection in erythcrocyte (CAP-e) assay was performed of the serum, according to the method of Honzel and colleagues (*15*), to see if the antioxidant compounds in açai were capable of providing cellular protection. The results reflect the antioxidants in serum that are actually able to enter into living cells and thereby potentially contribute to a reduction of oxidative damage within the cell. The CAP-e assay were compared to the thiobarbituric acid reactive substances (TBARS) assay which measures the rate of lipid peroxidation (*15*).

In the randomized clinical trial, blood samples at baseline, 1-hour, and 2-hours following consumption of the juice or placebo were tested for antioxidant capacity using several antioxidant assays as well as the TBARS assay. A within subject post consumption comparison showed an increase in serum antioxidants at 1-hour ($p<0.03$) and 2-hours ($p<0.015$), as well as inhibition of lipid peroxidation at 2-hours ($p<0.01$) (*16*).

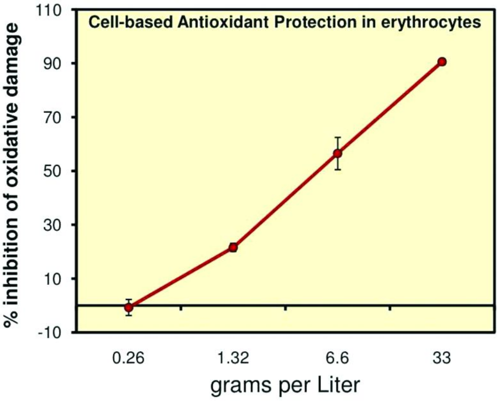

Figure 1. Cell-based antioxidant protection of açai.

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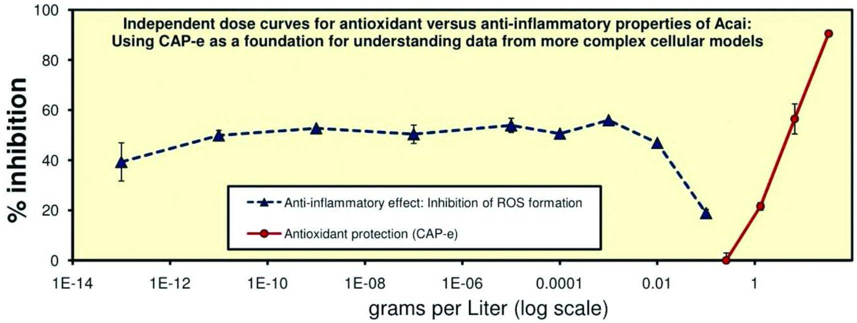

Figure 2

Study of the Pulp's Anti-Inflammatory and Immune Properties

The demonstration that açai pulp contained biologically relevant antioxidant compounds encouraged further study of its biological activity to determine whether it had anti-inflammatory properties.

Açai pulp showed a significant dose dependent inhibition of lipopolysaccharides (LPS)-induced nitric oxide (NO) production in several different assay systems, in the range of 250-2500 µg/ml (*14*). Since inhibition of LPS-induced NO has been correlated with anti-inflammatory activity, the results suggested that açai might serve as an anti-inflammatory food of interest to those studying allergic, inflammatory and auto-immune disorders.

With the further discovery reported in 2006 that açai pulp had cyclooxygenase (COX) inhibitory properties (*14*), an investigation was carried out to determine if the pulp could increase phagocytic capacity of cells *in vitro*.

Neutrophils and macrophages play a significant role in phagocytosis during destruction of microbial invaders. Freeze-dried açai was found to increase macrophage activity at concentrations of 5-250 µg/ml *in vitro*, with an increase in the number of engulfed yeast per macrophage at 5 µg/ml (*14*). In contrast, the lymphocyte proliferation assay (LPA), a measure of immune activation and stimulation, showed that freeze-dried açai showed no significant direct effect on lymphocyte proliferation across a very wide concentration range of 5-1,000 µg/ml (*14*), which was a welcome finding in that it suggests the pulp does not contain mitogenic compounds.

Two of the most interesting biological effects of açai *in vitro* pointed to a potential for açai to provide multi-facetted anti-inflammatory support. The anti-inflammatory data revealed a strong inhibition in the formation of ROS in human polymorphonuclear cells under oxidative stress (*14*). The effect was seen at very low doses, and could not be accounted for by a direct antioxidant effect alone. The ROS inhibitory effect remained statistically significant at doses 10^{12} fold lower than the limit of detection for antiodixant protection of live cells by açai in the CAP-e assay (Figure 2). Additional evidence of açai's anti-inflammatory properties was the discovery of its strong and significant inhibition of PMN cell migration in response to the inflammatory mediator leukotriene B4.

Taken together, these results suggested to the authors that a pilot study should be conducted to measure its anti-inflammatory effects in older adults, to see if consuming an açai-based fruit juice twice daily over 12 weeks would have an affect on range of motion (ROM) and perceived pain associated with joint mobility of various anatomical areas along the vertical column, including neck, back, hip, and knees.

In Vivo Study on Inflammatory Conditions, Including Joint Pain

To evaluate the impact of oral consumption of an açai-rich fruit-and berry blend (Monavie Active®) on pain and range of motion (ROM), an open-label clinical pilot study was performed on subjects, 44 to 84 years of age. Many of the subjects had osteoarthritis, with limited ROM associated with varying degrees of pain that affected their quality of life.

The manufacturer of the juice had not removed the water insoluble fiber found in Açai pulp, hence the acai pulp was not filtered to remove this "sediment" as is commonly done to clarify commercial açai juices. Mertens-Talcott and colleagues reported that the insoluble fraction in açai pulp contains an appreciable amount of the fruit's polyphenols, particularly anthocyanins, and by way of a pharmacokinetic study of healthy adults showed significant differences in antioxidant levels in serum following consumption of either clarified or non-clarified açai pulp juice (*17*).

In the ROM study of the açai juice blend, subjects were assessed at baseline and at 2, 4, 8, and 12 weeks by structured nurse interviews, questionnaires on pain and activities of daily living (ADL), blood samples, and ROM assessment. Pain was scored using a visual analogue scale (VAS), while ROM assessment was performed using dual digital inclinometry according to American Medical Association (AMA) guidelines. Consumption of the juice by the subjects resulted in pain reduction ($p<0.01$) (Figure 3), improved ROM ($p<0.05$), and improved ADL ($p<0.025$) (*18*).

To evaluate antioxidant uptake in this older population with some level of chronic inflammatory conditions (in contrast to the younger and healthier population used for the initial study of acute antioxidant uptake), we used the CAP-e assay according to the method of Honzel et al (*15*). The CAP-e assay showed a significant improvement in antioxidant status at 2 weeks ($p<0.05$), which kept improving throughout the 12 weeks of study participation (*18*) (Figure 4). The most significant outcome was individual improvements in ROM of both knees in subjects as a group ($p<0.0001$), as well as improvement in lumbar spine ROM ($p<0.01$). Interestingly, the serum antioxidant status showed an inverse correlation with both pain levels and reduced joint mobility. Nurses records of conversations with subjects revealed that as their ROM improved they took on more tasks resulting in increased pain as shown on the VAS.

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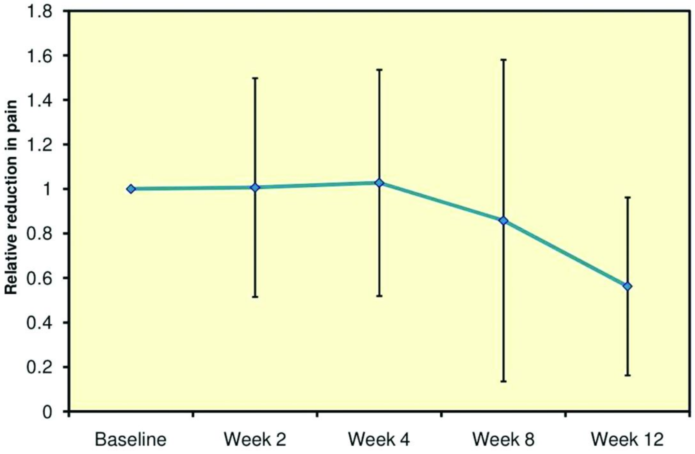

Figure 3. Reduction in perceived pain: Visual analog scale.

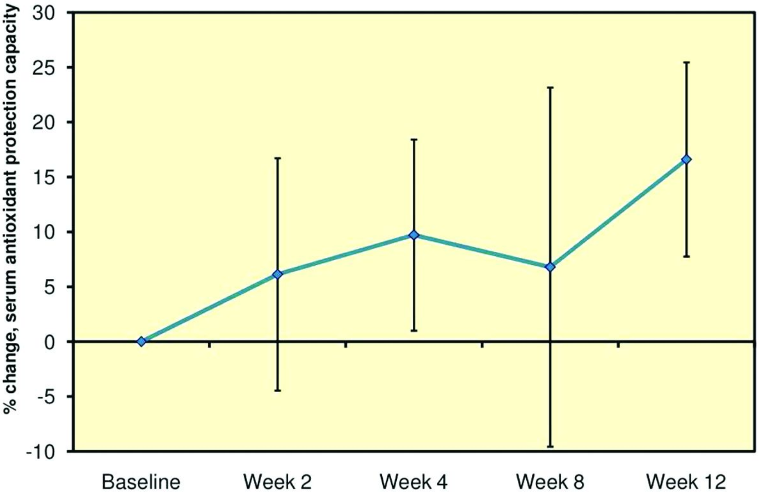

Figure 4. Serum antioxidant protection capacity testing in the CAP-e assay.

These results in humans suggest that antioxidants in açai pulp are bioavailable, and that the additional anti-inflammatory properties derived from compounds in the açai-based juice might allow for normal immune surveillance while simultaneously reducing inflammatory activity *in vivo*.

Further research on açai pulp *in vivo* is warranted to determine how wide a range of biological effects this fruit may have in humans and to elucidate its mode of action and range of potential benefits. Given the stability of the pulp in various food applications as shown by food chemists (*19*, *20*), there is a high probability that açai pulp as a food ingredient will be found in a growing number of juice products and other foods in the future. However, to determine whether such açai-fortified food products actually benefit human health, the products themselves should be tested to substantiate any health claims made for them.

References

1. Cavalcante, P. B.; Johnson, D. Edible Palm Fruits of the Brazilian Amazon. In *Iconography of the Most Important Spontaneous and Cultivated Plants from the Amazon Region*; Calavante, P. B., Ed.; 1977; p 93.
2. Chaves, J. M.; Pechnik, E. *O Açai, um dos alimentos basicos da Amazonia*; 1945; as referenced by Yuama, L. K. O.
3. Schauss, A. G.; Wu, X.; Prior, R. L.; Ou, B.; Patel, D.; Huang, D.; Kababick, J. P. Phytochemical and nutrient composition of the freeze-dried Amazonian palm berry, *Euterpe oleraceae* Mart. (açai). *J. Agric. Food Chem.* **2006**, *54*, 8598–8603.
4. Roosevelt, A. C.; Lima de Costa, M.; Lopes Machado, C.; Michab, M.; Mercier, N.; Valladas, M. H.; Feathers, J.; Barnett, W.; Imazio da Silveira, M.; Henderson, A.; Silva, J.; Chernoff, B.; Reese, D. S.; Holman, J. A.; Toth, N.; Schick, K. Paleoindian cave dwellers in the Amazon: The peopling of the Americas. *Science* **1996**, *272*, 373–384.
5. McCann, J. M. Before 1492: The making of the pre-Colombian landscape. Part II: The vegetation and implications for restoration for 2000 and beyond. *Ecol. Restor.* **1999**, *17*, 107–119.
6. Schauss, A. G. *Açai: An Extraordinary Antioxidant-Rich Palm Fruit from the Amazon*; Biosocial Publications: Tacoma, 2009; pp 31−38.
7. Beaglehole, J. C. *The Endeavor Journal of Joseph Banks, 1768−1771*; Angus & Robertson: Wellington, 1962; Vol. 1, pp 200−201.
8. Edwards, W. H. *Voyage Up the River Amazon, Including Residence at Para in 1846*; The Narrative Press: Santa Barbara, CA, 2004; p 28, original edition published by D. Appleton & Company, New York, 1848.
9. Weinstein, S.; Moegenburg, S. Açai palm management in the Amazon estuary: Course for conservation or passage to plantations?. *Conserv. Soc.* **2004**, *2*, 315–346.
10. Wu, X.; Gu, L.; Prior, R. L.; McKay, S. Characterization of anthocyanins and proanthocyanidins in some cultivars of ribes, aronia, and sambucus and their antioxidant capacities. *J. Agric. Food Chem.* **2004**, *52*, 7846–7856.
11. Wu, X.; Beecher, G. R.; Holden, J. M.; Haytowitz, D. B.; Gebhardt, S. E.; Prior, R. L. Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. *J. Agric. Food Chem.* **2004**, *52*, 4026–4037.
12. Wu, X.; Prior, R. L. Systematic identification and characterization of anthocyanins by HPLC-ESI-MS/MS in common foods in the United States: Fruits and berries. *J. Agric. Food Chem.* **2005**, *53*, 4852–4860.

Downloaded by COLUMBIA UNIV on June 26, 2012 | http://pubs.acs.org
Publication Date (Web): March 18, 2010 | doi: 10.1021/bk-2010-1035.ch013

13. Pozo-Insfran, D. D.; Percival, S. S.; Talcott, S. T. Açai (*Euterpe oleracea* Mart.) polyphenolics in their glycoside and aglycone forms induce apoptosis of HL-60 leukemia cells. *J. Agric. Food Chem.* **2006**, *54*, 1222–1229.
14. Schauss, A. G.; Wu, X.; Prior, R. L.; Ou, B.; Huang, D.; Owens, J.; Agarwal, A.; Jensen, G. S.; Hart, A. N.; Shanbrom, E. Antioxidant capacity and other bioactivities of the freeze-dried Amazonian palm berry, *Euterpe oleraceae* Mart. (açai). *J. Agric. Food Chem.* **2006**, *54*, 8604–8610.
15. Honzel, D.; Carter, S. G.; Redman, K. A.; Schauss, A. G.; Endres, J.; Jensen, G. S. Comparison of chemical and cell–based antioxidant methods for evaluation of foods and natural products: Generating multifaceted data by parallel testing using erythrocytes and polymorphonuclear cells. *J. Agric. Food Chem.* **2008**, *56*, 8319–8325.
16. Jensen, G. S.; Wu, X.; Patterson, K. M.; Barnes, J.; Carter, S. G.; Scherwitz, L.; Beaman, R.; Endres, J. R.; Schauss, A. G. *In vitro* and *in vivo* antioxidant and anti-inflammatory capacity of an antioxidant-rich fruit and berry juice blend. Results of a pilot and randomized, double-blind, placebo-controlled, crossover study. *J. Agric. Food Chem.* **2008**, *56*, 8326–8333.
17. Mertens-Talcott, S. U.; Rios, J.; Jilma-Stohlawetz, P.; Pacheco-Palencia, L. A.; Meibohm, B.; Talcott, S. T.; Derendorf, H. Pharmacokinetics of anthocyanins and antioxidant effects after the consumption of anthocyanins-rich açai juice and pulp (*Euterpe oleracea* Mart.) in human healthy volunteers. *J. Agric. Food Chem.* **2008**, *56*, 7796–7802.
18. Jensen, G. S.; Schauss, A. G.; Beaman, R.; Ager, D. M. Açai fruit (*Euterpe oleracea* Mart): Systematic and collaborative study of the phytochemistry, nutrient composition, and *in vitro* and *in vivo* bioactivities of the Amazonian palm fruit in humans. *Altern. Ther. Health Med.* **2009**, *15*, S90–S91.
19. Bobbio, F. O.; Bobbio, P. A.; Oliveira, P. A.; Fadelli, S. Stability and stabilization of the anthocyanins from *Euterpe oleracea* Mart. *Acta Aliment.* **2005**, *31*, 371–377.
20. Pacheco-Palencia, L.; Hawken, P.; Talcott, S. T. Phytochemical, antioxidant and pigment stability of açai (*Euterpe oleracea* Mart.) as affected by clarification, ascorbic acid fortification, and storage. *Food Res. Int.* **2007**, *40*, 620–628.

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Chapter 14

Using *Caenorhabditis elegans* To Study Bioactivities of Natural Products from Small Fruits

Linking Bioactivity and Mechanism *in Vivo*

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Natural products from small fruits elicit a variety of bioactivities studied primarily *in vitro*. Although rigorous, these approaches fail to replicate the inherent complexity of the whole organism, where uptake and detoxification processes limit interactions with the target of interest. Currently, most *in vivo* studies use rodent models, such as mice and rats. Invertebrates, such as *Caenorhabditis elegans* nematodes, offer an efficient alternative to rodents. *C. elegans* nematodes are cheap to maintain, easily cultivated in large numbers and amenable to genetic and cellular studies. This chapter provides a brief introduction to the use of *C. elegans* for *in vivo* studies of natural products. In addition, we describe our own use of *C. elegans* to elucidate bioactivities of blueberry proanthocyanins and a collection of resveratrol analogs, with the goal of encouraging new investigations of natural products using *C. elegans*.

There is great interest in potential health benefits of natural products. Small fruits are particularly rich sources of promising compounds. Realizing this promise will require efficient innovative approaches for identifying and characterizing natural product bioactivities. The free-living nematode,

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Caenorhabditis elegans, provides a powerful, but underutilized, resource for such investigations. The power of the *C. elegans* model is illustrated by the fact that major cellular processes, such as apoptosis and microRNA-based gene regulation, were originally described in this organism.

Introduction to *Caenorhabditis elegans*

Laboratory Equipment and Supplies

C. elegans is within the *Rhabditidae* family of free-living nematodes that feed on bacteria and slime molds. In the laboratory, *C. elegans* populations can be easily grown on solid agar medium supplemented with a food source of *E. coli* bacteria. In fact, growth and maintenance of *C. elegans* in the laboratory requires minimal investment in specialized materials and equipment, other than for items common to a standardly-equipped biological laboratory (Table 1). The animals grow well within the range of temperatures between 15°C to 25°C. Many studies can be conveniently conducted at room temperatures of 20-23°C, if controlled within this range. Otherwise, a refrigerated incubator set at the desired temperature provides a standardized cultivation environment. Nematodes are usually cultivated on solid agar medium in plastic petri dishes with a lawn of *E. coli* bacteria as a food source (*1*). Methodologies for growing large *C. elegans* populations in solid and liquid media have been developed (*2*, *3*). These conditions may be suitable for studies of natural products. In addition, several Internet resources are now available for obtaining information on a variety of aspects of *C. elegans* biology and methodology (Table 2).

Anatomy

The *C. elegans* anatomy contains most major tissues and cell types found in mammals. The cylindrical body is radially symmetric. A collagenous cuticle surrounding the body protects against abrasion and dehydration. The cuticle is open to the environment at the head for food ingestion and sensation, at the vulva for egg laying and at the anus for defecation and sensation. The cuticle is synthesized by epidermal cells that lie just beneath it. Interspersed within the epidermal layer are four longitudinal rows of body wall muscles that promote locomotion and movement.

In the head, the major medial structures are the pharynx, the head nerve ganglia and nerve ring. The posterior end of the pharynx joins the intestine, which proceeds for the length of the body to the anus. Food is drawn into the body by the pumping action of the pharynx muscles (*4*). Pharynx muscles also concentrate the food particles, expeling excess liquid, and crush food particles for passage to the intestine. Within the intestine, food is further broken down and the components are absorbed.

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Table 1. Equipment and Supplies Needed for *C. elegans* Research

Item	*Purpose*	*Approximate Cost*
Refrigerated incubator	Growth and maintenance of worm strains within appropriate temperature range (15°-25°C)	$4,000
Stereodissecting microscope	Visualizing worms for manipulation and phenotypic scoring	$3,000
Glass pasteur pipets, platinum wire, alcohol lamps, wooden toothpicks	Supplies for manipulating worms during strain maintenance and growth	$200
Growth medium and petri dishes	Worm growth	≤ $1.00/ plate
Autoclave, clinical centrifuge, refrigerator	Standard laboratory equipment for media preparation, worm collection	*
Ultra low temperature freezer	Long-term storage of worm strains	*

* Costs not included for equipment considered to be standard for biological research.

Table 2. *Caenorhabditis elegans* Internet Resources

Title	*URL*	*Resources*
Caenorhabditis Genetics Center	www.cbs.umn.edu/CGC/	*Caenorhabditis* strain collection
WormBase	www.wormbase.org	Curated *C. elegans* Genome resource
Caenorhabditis elegans WWW Server	elegans.swmed.edu	Links to many *C. elegans*-related websites
Worm Book	www.wormbook.org	Worm biology and methodology articles
Worm Atlas	www.wormatlas.org	Detailed *C. elegans* anatomical descriptions
Worm Classroom	www.wormclassroom.org	Basic *C. elegans* methodology information

The hermaphrodite nervous system consists of 302 neurons, which utilize most of the major neurotransmitters, including acetylcholine, GABA, serotonin and dopamine (*5*). The nervous system allows the animal to perform several well-characterized quantifiable behaviors, such as locomotion, chemotaxis, mechanosensation and foraging. The male nervous system contains additional neurons which regulate male mating behavior (*6*).

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The gonad consists of somatic and germline structures. *C. elegans* are hermaphroditic, with the adult hermaphrodite producing both sperm and oocytes for self-fertilization. In addition, X-chromosome non-disjunction results in XO zygotes which develop into males that produce sperm for cross-fertilization.

The hermaphrodite gonad consists of two symmetric U-shaped arms which begin at the vulva, extend towards the head and tail and then reflex back toward the midbody. The distal end of each arm contains the germline stem cells which divide and differentiate progressively along the length of each gonad arm. Spermatogenesis occurs during late larval development and the gonad switches to oogenesis during adulthood. Fertilization occurs in the spermatheca, where the sperm are collected, when a mature oocyte is extruded from the proximal gonad. The fertilized egg passes from the spermatheca into the uterus. Within a few hours of fertilization, a protective eggshell matures around each embryo and the eggs are passed out of the vulva and laid.

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The *C. elegans* Life Cycle

C. elegans development proceeds through four distinct larval stages (Figure 1). Between each larval stage, the prior-stage cuticle is shed. Cuticle shedding occurs during the inter-molt lethargus when feeding and movement cease. Developmental age can be determined by the presence of stage-specific anatomical features. Such developmentally informative features include the presence or absence of cuticular alae, which are longitunial ridges in the cuticle that are present in first-stage L1 larvae, dauer larvae and adults. Gonadal development can also be a useful marker of developmental age, particularly during the early larval stages. Late-stage larvae can be identified by the presence of a developing vulva. Following the 4th larval molt, mature adults emerge with a fully-differentiated vulva at the midbody. Additionally, the adult gonad is mature and reproduction may commence.

In the presence of signals reflecting environmental conditions unsuitable for continued reproduction, larvae enter an alternative developmental pathway to form dauer larvae, which arrest development as an alternative third larval stage (*7*). The environmental triggers for dauer diapause include limited food availability, high population density signaled by high levels of a constitutively-secreted pheromone, and high temperature. These cues may only trigger dauer arrest if detected in the first larval stage (L1). If appropriately detected, then L2 larvae develop into a special "predauer" form, called the L2d, which molts into the dauer larvae. Dauer larvae possess specialized features that promote survival and dispersal, such as plugged cuticle openings to prevent dehydration, adaptive behaviors to enhance dispersal, and upregulation of antioxidant and heat shock proteins. In addition to increased stress resistance, metabolism is shifted in dauer larvae to utiize fat preferentially. Dauer larvae may resume normal development, molting into the fourth larval stage, upon transfer to a more hospitable environment.

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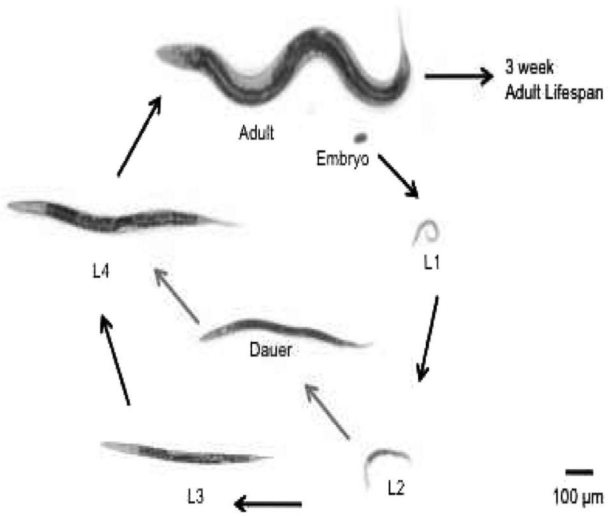

Figure 1. The C. elegans life cycle. Embryonic development occurs in the egg until a mature first stage larva (L1) hatches. Larval development proceeds through four larval stages (L1-L4) before the final molt into the adult hermaphrodite. Adults reproduce and lay eggs for 3-5 days, then enter a postreproductive phase for approximately 2 weeks before death. Inhospitable environments trigger developmental arrest at the dauer larval stage. Dauer larvae are long-lived and stress resistant. Dauers resume development as L4s after return to favorable conditions.

Genetic Analysis in *C. elegans*

The *C. elegans* genome was the first metazoan genome to be fully sequenced (*8*). The genome annotations have been compiled into Wormbase (www.wormbase.org). Wormbase provides a wealth of information about gene conservation, structure and function in this organism. In addition, information obtained from standard forward genetic analysis is also included. Wormbase provides a comprehensive resource on *C. elegans* biology for current knowledge about a protein or pathway of interest.

Several of the signaling pathways that are important for human development or disease are conserved in *C. elegans*. For example, Notch signaling regulates several cell fate decisions in the embryo and developing larvae (*9*, *10*). A *C. elegans* homolog of mammalian insulin/IGF-I receptors, together with a p110 PI3K, regulate dauer arrest and adult longevity (*11*). The Ras GTPase homolog, LET-60, couples signaling by FGF- and EGF-related receptors to intracellular MAPK pathways to regulate a variety of developmental events (*12*). The Nrf-family transcription factor, SKN-1, carries out dual functions in embryonic mesodermal specification and oxidative stress resistance in larvae and adults (*13*). Natural products interacting with any of these, or other, conserved signaling pathways should produce visible phenotypes that may constitute the basis for further mechanistic study.

Considerations for Studying Natural Products in *C. elegans*

A variety of approaches are available for *in vivo* studies of natural products in *C. elegans*. Usually, the first goal is to identify quantifiable phenotypes that result from treatment with the compounds of interest. Vehicle controls are essential. Some examples of easily-scored *C. elegans* phenotypes include embryonic lethality, constitutive dauer arrest, sterility, egg-laying defects, locomotory defects and changes in adult survival. Many investigators begin by screening for phenotypes affecting biological processes within their areas of expertise. For example, this laboratory's specialization in the biology of *C. elegans* adults informs our studies of adult longevity and stress resistance after treatment with small fruit botanicals. However, any strong, scorable phentoype is useful for follow-up studies. The ease of working with *C. elegans* makes it feasible to screen compounds for a variety of phenotypes, without particular bias to specific processes.

The thick cuticle presents a major challenge for chemical interventions in *C. elegans*. This permeability barrier can make the effective dose in *C. elegans* many times higher than that needed for cultured cells. Our investigations usually involve chronic treatment with compounds supplemented in the growth medium. We prefer to use solid growth medium whenever possible, due to the ease of worm manipulation and visualization. On solid medium, compounds may enter the body through an oral route during food uptake. Exposure can also occur through contact with the sensory cilia, which are nerve terminals open to the environment through pores in the cuticle. At least one group of compounds likely acts by altering the function of these neurons (*14*). Exposure efficiency might be increased by the use of genetic or environmental conditions which weaken the cuticle, although this area has not been heavily explored.

One problem with delivering treatments on solid medium is inefficient delivery due to uncertain availability in the agar surface. Some of these concerns can be alleviated by using liquid growth conditions, rather than solid agar. One approach is to use a defined worm culture medium, with necessary nutrients added from purified sources (*3*, *15*). Alternatively, a standard buffer (S buffer) can be used as the medium and heat-killed bacteria, at a standardized concentration, can be provided as a food source (*2*). Natural products in the medium may be more efficiently ingested under liquid conditions due to the increased exposure of the worms. However, phenotypic characterization can be more difficult than on solid medium. Fungal and bacterial contamination can also be more serious problems when growing worms under liquid conditions.

An additional concern for both solid and liquid medium is the possibility that natural products will be subject to metabolic degradation by the bacteria provided as a food source. To alleviate this problem, the bacteria can be neutralized, either by UV irradiation or antibiotic treatment. Secondary effects on bacterial metabolism have been determined to affect some worm phenotypes (*16*).

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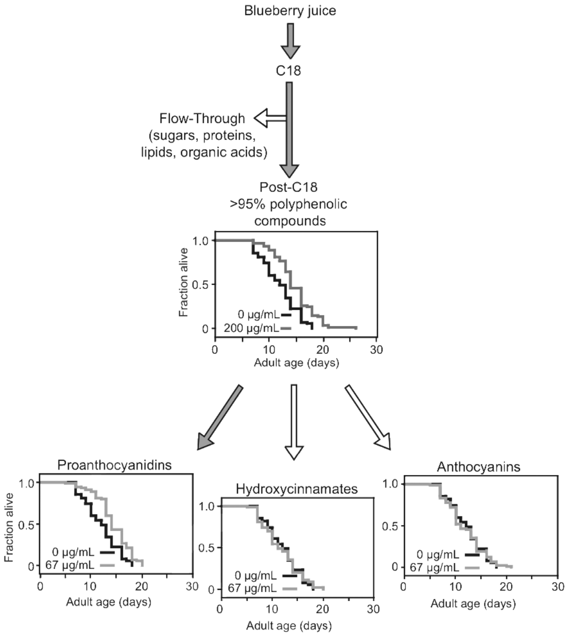

Figure 2. The prolongevity activity of blueberry polyphenols cofractionated with proanthocyanidins. Total blueberry polyphenols, collected in the C18 eluate of blueberry juice, extended C. elegans adult lifespan (0 µg/ml, mean lifespan 12.0 days, n=97; 200 µg/ml, mean lifespan 14.8 days, n=88, p vs 0 µg/ml <0.0001). The total polyphenol mix is composed of nearly equal masses of 3 main components: proanthocyanidins, anthocyanins and hydroxycinnamates. Only the proanthocyanidin fraction retained prolongevity activity (proanthocyanidins (67 µg/ml) mean lifespan 14.4 days, n=98, p vs 0 µg/ml <0.0001; hydroxycinnamates (67 µg/ml) mean lifespan 11.7 days, n= 91, p=0.9; anthocyanins (67 µg/ml) mean lifespan 11.7 days, n= 99, p=0.96). Adapted from reference (1).

In Vivo Studies of Small Fruit Natural Products in *C. elegans*

Our laboratory has studied several compounds found in small fruits for their effects on *C. elegans* adults. The phenotypes we have examined include adult survival, stress resistance and the growth of germline tumors in *gld-1* mutants. These studies have revealed that natural products can have a range of effects, from beneficial to detrimental. This phenotypic diversity appears to primarily reflect structural differences in the compounds. Thus, small fruits contain compounds with the potential to interact with *in vivo* targets on a structurally-specific basis. This work provides examples of the types of information that can be gained from characterizing natural products *in vivo* using *C. elegans*.

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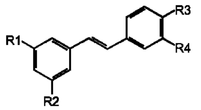

		R1	R2	R3	R4
(1)	Resveratrol	OH	OH	OH	
(2)	Piceatannol	OH	OH	OH	OH
(3)	Pinostilbene	OH	OCH_3	OH	
(4)	Desoxyrhapontigenin	OH	OH	OCH_3	
(5)	3-OH-5,4'-dimethoxystilbene	OH	OCH_3	OCH_3	
(6)	Pterostilbene	OCH_3	OCH_3	OH	
(7)	Resveratrol-trimethylether	OCH_3	OCH_3	OCH_3	

	Survival	Toxicity	Tumor growth inhibition	Bio-availability
(1)	1.09*	0.92	1%	n.t.
(2)	1.12	0.89	n.t.	n.t.
(3)	1.08	0.93	n.t.	n.t.
(4)	0.75**	1.33	11%**	32%
(5)	0.47*	2.13	n.t.	n.t.
(6)	0.74**	1.35	10%**	83%
(7)	0.66**	1.52	11%*	21%

Figure 3. Comparison of stilbene bioactivities in C. elegans. Top, stilbene structure showing C3 (R1), C5 (R2), C4' (R3) or C5' (R4) positions. Lower left, stilbenes examined in this study. Lower right, stilbene bioactivies at 100 μM. Numbers refer to compounds as shown on left. Survival is mean lifespan relative to untreated control averaged from 2-4 independent trials. Toxicity is the inverse of the survival measurement. Antitumor activity is percent reduction in tumor size after 96 hours of treatment, relative to untreated control. Value is the average of 3-7 independent trials for each stilbene. Bioavailability is expressed as percent steady state level measured in worms treated for 48 hours with 100 μM dose of indicated stilbene. Value determined from the average of 3-4 independent samples. Significance was determined by paired t-test ($p<0.05$, ** $p<0.001$); n.t., not tested. Adapted from reference (29).*

Blueberry Polyphenols Enhance Longevity and Stress Resistance of *C. elegans* Adults

Blueberry fruit contains high levels of polyphenolic compounds with health-related activities, such as the ability to scavenge free radicals *in vitro*. Blueberry polyphenols have also shown benefits in alleviating age-related cognitive and behavioral deficits in rodents (*17*). The rodent supplementation studies were relatively short-term, which may limit the effect magnitude. The short *C. elegans* lifespan of 2-3 weeks allowed us to study the effects of life-long treatment with blueberry polyphenols (*18*).

Growth of *C. elegans* on blueberry polyphenol-supplemented solid medium increased mean adult lifespan by 28% and maximum lifespan by 14%. While this effect was less dramatic than that of some gene mutations, such as *daf-2*, it was still significant and robust. This treatment also enhanced survival under thermal stress (35°C), but did not protect against oxidative stress from paraquat treatment or due to the *mev-1(bn1)* gene mutation. This selective effect of blueberry polyphenols on stress resistance suggests the possibility that protection against aging-related stress involves factors that overlap with thermal stress, but not with oxidative stress.

The blueberry polyphenol mixture could be fractionated into its three major subcomponents of proanthocyanidins, anthocyanins and hydroxycinnamic acids. Treatment with each subfraction showed that the prolongevity effect cofractionated with the proanthocyanidins (Figure 2). All three components are

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reported to possess similar free-radical scavenging activities *in vitro*, suggesting that the benefits of proanthocyanin treatment result from effects other than antioxidant activity. The proanthocyanidins consist of different-length polymers of catechin monomers. Size-based fractionation of the proanthocyanins indicated that the high-molecular-weight proanthocyanins were more active than the low-molecular weight polymers in this activity. Consistent with this, preliminary results have indicated that the catechin monomers alone do not enhance *C. elegans* longevity (Wilson, M.A., Wolkow, C.A., unpublished).

Adult lifespan and stress resistance in *C. elegans* are regulated by several genetic pathways. This allowed us to use *C. elegans* genetics to identify candidate targets for the blueberry effects. If a gene is involved in the pathways by which blueberry polyphenols extend adult lifespan, than mutations disrupting that gene's function would block blueberry's ability to extend lifespan. Similarly, we could conclude that a gene was not involved in the blueberry phenotypes if blueberry treatment could still extend lifespan of mutants lacking that gene.

A number of candidate pathways were examined in this way. We discovered that mutations interfering with the function of a CaMKII signalling pathway blocked lifespan extension in blueberry-treated animals. This pathway includes UNC-43/CaMKII, NSY-1/MAPKKK and SEK-1/MAPKK (*19*). Components of this pathway also regulate pathogen response in *C. elegans* (*20*). It is possible that blueberry polyphenols may stimulate signaling through this pathway, possibly by altering calcium homeostasis to impact UNC-43/calmodulin kinase function. A role for blueberry polyphenols in altering calcium homeostasis has been elucidated in studies of mammalian rodent cells in culture (*21*, *22*). Alternatively, blueberry polyphenols could enhance the function of downstream products of this pathway. In this scenario, CaMKII pathway defects would block the production of these products, thus eliminating the target of action for blueberry polyphenols.

Structure-Activity Relationships for Stilbene Bioactivities in *C. elegans*

Stilbenes are a class of polyphenol compounds produced by plants under stressful conditions. A variety of stilbenes have been characterized that differ by the presence of hydroxylation or methoxylation modifications at the C3, C5 and C4' positions (Figure 3). In a variety of systems, such modifications have been shown to affect bioactivity. Changes in bioactivity may reflect differing bioavailability or altered target interactions.

One particularly well-studied stilbene is resveratrol, which is 3,4',5-hydroxylated and non-methoxylated. Resveratrol is produced by a variety of small fruits, including grapes. *In vitro* studies have documented resveratrol's antioxidant activity (*23*). *In vivo*, resveratrol antagonizes the peroxiation of fatty acids and membrane lipid, potentially via this antioxidant action (*24*). Furthermore, resveratrol supplementation can protect rodents from the detrimental effects of high-fat diet (*25*). Resveratrol also has prolongevity effects in wide range of organisms, from yeast to worms to flies (*26*). This activity is due, at least in part, to stimulation of the sirtuin protein deacetylases. Finally, resveratrol has shown anti-cancer effects in *in vitro* systems (*23*).

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A related compound, pterostilbene, is dimethoxylated at the C3 and C5 positions and hydroxylated at the C4' position. Similar to resveratrol, pterostilbene also displays antioxidant and anticancer activities (*27*). In side-by-side comparisons, resveratrol and pterostilbene showed similar effectiveness in growth inhibition towards a mouse mammary gland cancer cell (*27*). However, resveratrol inhibited COX oxygenase activity more strongly than pterostilbene (*27*). A third stilbene, resveratrol-trimethylether (also called trimethoxystilbene), is trimethoxylated at the C3, C4' and C5 positions. Interestingly, trimethoxystilbene caused a stronger inhibition of cancer cell growth *in vitro* than resveratrol and pterostilbene, which showed similar growth inhibition activity in this study (*28*).

In order to better understand how methoxylation and hydroxylation affect stilbene bioactivity *in vivo*, we compared seven different stilbenes in *C. elegans* (*29*). First, the toxicity profile for each stilbene was examined. In general, methoxylation was associated with greater toxicity than hydroxylation. Stilbenes with hydroxylated or unmodified positions, such as resveratrol or pinostilbene, were either non-toxic or modestly beneficial to the survival of adult *C. elegans*. Highly-methoxylated stilbenes, such as resveratrol-trimethylether and dimethoxystilbene, were the most toxic and shortened adult lifespan by approximately 40%. Intermediate toxicity was observed for stilbenes with fewer methoxylated positions, such as pterostilbene and desoxyrhapontigenin.

All somatic cells in the *C. elegans* adult are postmitotic and terminally-differentiated. Thus, stilbene toxicity reflects effects on cell survival and function, but not on cell proliferation. To investigate the effects of each stilbene on cell proliferation *in vivo*, a *C. elegans* cancer model was utilized. In wildtpe worms, the *gld-1* gene controls germline proliferation within the gonad. The *gld-1(q485)* mutation interferes with this function and results in inappropriate germline growth (*30*). This phenotype is visible as a germline tumor which fills the body. Thus, we could determine the *in vivo* anti-proliferation activities of these stilbenes by measuring the relative sizes of germline tumors in *gld-1(q485)* animals after treatment. This analysis showed that methoxylated stilbenes had more potent effects in reducing *gld-1* tumor size than hydroxylated stilbenes. Thus, methoxylation appeared to enhance stilbene bioactivity, relative to hydroxylation, for both adult survival and *gld-1* tumor growth.

The mechanisms behind the enhanced *in vivo* bioactivity of methoxylated stilbenes are not known. One possibility is that increased bioactivity reflects greater bioavailability *in vivo*. Stilbenes are subject to modification by xenobiotic glucuronidation or sulfonation processes which increase their solubility and facilitate excretion. Methoxylation can retard these processes, protecting the compound from modification and excretion, thereby extending the time for target interactions. To begin investigating this possibility, the levels of three stilbenes was measured in worms after a 48 hour treatment at 100 μM in solid agar. Under these conditions, the treated and washed worms contained similar levels of all three stilbenes. This indicated that uptake was not dramatically different among the three stilbenes tested, although they differed somewhat in their lethality. It should be noted that the treated worms contained unexpectedly high levels of stilbenes, suggesting that the compounds were extremely well absorbed in these experiments. Further characterization of uptake parameters for these compounds is necessary to validate these measurements. In addition, future experiments to identify the detoxification pathways limiting stilbene bioavailability in *C. elegans* will help to clarify the phenotypic differences between methoxylated and hydroxylated stilbenes.

Summary

This chapter describes a few approaches for using *C. elegans* for studies of small fruit natural products, based on experiments from our laboratory. Their short life cycle, amenability to genetic and cellular analysis, and translucent anatomy make *C. elegans* an accessible experimental system for new users. Thus, studies using *C. elegans* provide a low cost and efficient method for studying the effects of natural products *in vivo* in a living organism.

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References

1. Brenner, S. *Genetics* **1974**, *77*, 71–94.
2. Fabian, T. J.; Johnson, T. E. *J. Gerontol.* **1994**, *49*, B145–56.
3. Szewczyk, N. J.; Kozak, E.; Conley, C. A. *BMC Biotechnol.* **2003**, *3*, 19.
4. Avery, L.; Shtonda, B. B. *J. Exp. Biol.* **2003**, *206*, 2441–57.
5. Sulston, J. E. *Cold Spring Harb. Symp. Quant. Biol.* **1983**, *48* (Pt. 2), 443–52.
6. Barr, M. M.; Garcia, L. R. *WormBook* **2006**, 1–11.
7. Hu, P. J. *WormBook* **2007**, 1–19.
8. *Science* **1998**, *282*, 2012–8.
9. Priess, J. R. *WormBook* **2005**, 1–16.
10. Greenwald, I. *WormBook* **2005**, 1–16.
11. Gami, M. S.; Wolkow, C. A. *Aging Cell* **2006**, *5*, 31–7.
12. Sundaram, M. V. *WormBook* **2006**, 1–19.
13. An, J. H.; Blackwell, T. K. *Genes Dev.* **2003**, *17*, 1882–93.
14. Collins, J. J.; Evason, K.; Pickett, C. L.; Schneider, D. L.; Kornfeld, K. *PLoS Genet.* **2008**, *4*, e1000230.
15. Houthoofd, K.; Braeckman, B. P.; Lenaerts, I.; Brys, K.; De Vreese, A.; Van Eygen, S.; Vanfleteren, J. R. *Exp. Gerontol.* **2002**, *37*, 1371–8.
16. Saiki, R.; Lunceford, A. L.; Bixler, T.; Dang, P.; Lee, W.; Furukawa, S.; Larsen, P. L.; Clarke, C. F. *Aging Cell* **2008**, *7*, 291–304.
17. Lau, F. C.; Shukitt-Hale, B.; Joseph, J. A. *Neurobiol. Aging* **2005**, 128–3226 Suppl 1.

18. Wilson, M. A.; Shukitt-Hale, B.; Kalt, W.; Ingram, D. K.; Joseph, J. A.; Wolkow, C. A. *Aging Cell* **2006**, *5*, 59–68.
19. Tanaka-Hino, M.; Sagasti, A.; Hisamoto, N.; Kawasaki, M.; Nakano, S.; Ninomiya-Tsuji, J.; Bargmann, C. I.; Matsumoto, K. *EMBO Rep.* **2002**, *3*, 56–62.
20. Kim, D. H.; Feinbaum, R.; Alloing, G.; Emerson, F. E.; Garsin, D. A.; Inoue, H.; Tanaka-Hino, M.; Hisamoto, N.; Matsumoto, K.; Tan, M. W.; Ausubel, F. M. *Science* **2002**, *297*, 623–6.
21. Joseph, J. A.; Carey, A.; Brewer, G. J.; Lau, F. C.; Fisher, D. R. *J. Alzheimers Dis.* **2007**, *11*, 433–46.
22. Joseph, J. A.; Neuman, A.; Bielinski, D. F.; Fisher, D. R. *J. Alzheimers Dis.* **2008**, *15*, 429–41.
23. de la Lastra, C. A.; Villegas, I. *Biochem. Soc. Trans.* **2007**, *35*, 1156–60.
24. Fremont, L. *Life Sci.* **2000**, *66*, 663–73.
25. Baur, J. A.; Pearson, K. J.; Price, N. L.; Jamieson, H. A.; Lerin, C.; Kalra, A.; Prabhu, V. V.; Allard, J. S.; Lopez-Lluch, G.; Lewis, K.; Pistell, P. J.; Poosala, S.; Becker, K. G.; Boss, O.; Gwinn, D.; Wang, M.; Ramaswamy, S.; Fishbein, K. W.; Spencer, R. G.; Lakatta, E. G.; Le Couteur, D.; Shaw, R. J.; Navas, P.; Puigserver, P.; Ingram, D. K.; de Cabo, R.; Sinclair, D. A. *Nature* **2006**, *444*, 337–42.
26. Wood, J. G.; Rogina, B.; Lavu, S.; Howitz, K.; Helfand, S. L.; Tatar, M.; Sinclair, D. *Nature* **2004**, *430*, 686–9.
27. Rimando, A. M.; Cuendet, M.; Desmarchelier, C.; Mehta, R. G.; Pezzuto, J. M.; Duke, S. O. *J Agric. Food Chem.* **2002**, *50*, 3453–7.
28. Pettit, G. R.; Grealish, M. P.; Jung, M. K.; Hamel, E.; Pettit, R. K.; Chapuis, J. C.; Schmidt, J. M. *J. Med. Chem.* **2002**, *45*, 2534–42.
29. Wilson, M. A.; Rimando, A. M.; Wolkow, C. A. *BMC Pharmacol.* **2008**, *8*, 15.
30. Francis, R.; Barton, M. K.; Kimble, J.; Schedl, T. *Genetics* **1995**, *139*, 579–606.

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Chapter 15

Characterization of Antioxidants in Nutmeg (*Myristica fragrans* Houttuyn) Oil

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Antioxidant capacity of nutmeg (*Myristica fragrans* Houttuyn) oil was investigated by the 2,2-diphenyl-1-picrylhydrazyl (DPPH$^\bullet$) free radical scavenging assay and the β-carotene-linoleic acid assay. The antioxidant EC_{50} values of the crude nutmeg oil dissolved in methanol were 2.4 μL/mL and 0.4 μL/mL, respectively. The former value was approximately equivalent to the free radical scavenging capacities of 462 μM BHT and 656 μM α-tocopherol, and the latter one was comparable to the inhibitive capacities of 43 μM BHT and 9 μM α-tocopherol against the oxidation of β-carotene and linoleic acid. Further investigations discovered three major antioxidant constituents of the nutmeg oil (i.e., eugenol, isoeugenol, and methoxyeugenol), which were sequentially separated and identified by silica open column chromatography, HPLC and GC-MS. Their antioxidant activities in the DPPH$^\bullet$ assay decreased in the following order: eugenol > methoxyeugenol > BHT > isoeugenol > α–tocopherol, while in the β-carotene-linoleic acid assay, the antioxidant activities of the chemicals were in the following order: α–tocopherol > BHT > isoeugenol > methoxyeugenol > eugenol.

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Introduction

Essential oils extracted from various herbs and spices have been widely used throughout history as cosmetics and aromatherapeutic agents as well as flavoring agents in food (*1*). Since it has been discovered that essential oils have many other biological properties such as antimicrobial, antioxidant, and anticancer activities, research on bioactive principles of essential oils has become increasingly popular (*1–4*). Nowadays many research groups are focusing their investigations on the pharmacological actions of essential oils from aromatic and medicinal plants (*3*, *5*, *6*). Among them, nutmeg (*Myristica fragrans* Houttuyn) oil is one of the most intensively studied objects because of its wide utilization in food and pharmaceutical industries (*7–9*).

Nutmeg oil, which is commercially obtained by steam distillation from kernels of nutmeg, usually contains more than 50 components. Nutmegs cultivated in tropical countries such as Indonesia and Sri Lanka often produce nutmeg oils in 10-15% of the seed weight (*10*, *11*). Regardless of some concerns about the toxicity of myristicin, elemicin, and safrole in nutmeg (*12*, *13*), nutmeg powder and its essential oil are still commonly and widely used as the spicy and flavoring agents in foods, and as medicinal agents (*7–9*, *14*, *15*).

Although nutmeg oil has been reported with various biological properties such as antimicrobial, antibacterial, antiplatelet, hepatoprotective, and hypolipidaemic effects (*8*, *9*, *16*, *17*), its antioxidant capacity has not been investigated thoroughly (*18–21*), let only the characterization of its inherent antioxidant constituents. Therefore, this study aimed to reveal the antioxidant capacity of nutmeg oil and its antioxidant components that were hypothesized to be linked with nutmeg's biofunctional activities. In this research, components with strong antioxidant activity were separated from the crude nutmeg oil through chromatographic techniques, and identified by high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS). In addition, the antioxidant capacities of nutmeg oil and its identified major constituents were assessed by two *in vitro* assays: DPPH$^{\bullet}$ free radical scavenging assay and β-carotene-linoleic acid assay.

Experimental

Materials

2,2-Diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) and α-tocopherol were purchased from Sigma Chemical Co. (St. Louis, MO). Eugenol (4-allyl-2-methoxy-phenol), isoeugenol (2-methoxy-4-propenyl-phenol), methoxyeugenol, myristicin, and silica gel (70-230 mesh, 60Å) were purhased from Aldrich Chemical Co. (Milwakee, WI). Nutmeg oil, linoleic acid, β-carotene, and butylated hydroxytoluene (BHT) were obtained from the Good Scents Co. (Oak Creek, WI), TCI (Portland, OR), Fluka Chemical Co (Milwakee, WI), and Acros (NJ), respectively. Tween 20 and all HPLC analytical grade solvents were from Fisher Scientific (Suwanee, GA).

Separation and Identification of Antioxidants from Nutmeg Oil

Separation by Silica Gel Open Column Chromatography

Six milliliters of the crude nutmeg oil dissolved in hexane was loaded on a column (60 cm × 2.5 cm) packed with silica gel (70-230 mesh, 60Å) and equilibrated with hexane. Then the sample was eluted by solvents sequentially in the following order: hexane (200 mL), hexane/DCM (1:1, 200 ml), DCM (200 mL), and DCM/methanol (1:1, 200 mL). The flow rate was controlled at 4 mL/min. Each collected fraction was in size of 7 mL.

Separation by Normal-Phase HPLC

The Spherisorb silica column (250 mm × 4.6 mm, 5 µm; Waters, MA, USA) was installed on a LC-10AT HPLC system (Shimadzu, Kyoto, Japan) and equilibrated with hexane. Fifty microliters of each fraction separated by the silica gel open column was injected into the HPLC column, and eluted with a linear gradient of a mixture solvent of DCM/methanol (1:1) at a flow rate of 1 mL/min. The absorbance of the eluant was scanned from 200 to 500 nm by a SPD-M10V photodiode array detector (Shimadzu, Kyoto, Japan).

Chemical Identification by Gas Chromatography−Mass Spectrometer (GC/MS)

To further separate and identify antioxidants from silica-HPLC, GC-MS (GC17A-QP 5050 MS) system (Shimadzu, Kyoto, Japan) installed with a DB-5 capillary column (60 m × 0.25 mm, thickness 0.25 µm; J&W Scientific, Folsom, CA, USA) was used. The oven temperature was programmed from 60°C to 280°C at 10°C/min and held at 280°C for 7 min. The injector and ion source temperatures were 220°C and 290°C, respectively. The detector voltage was 70 eV and the MS spectra were recorded in the mass range of m/z 43-350. Helium was used as a carrier gas and its flow rate was 1.1 mL/min. The injection volume was 1 µL and a split rate was 1:5. Identification of compounds was based on comparison with mass spectra and retention index (RI) of the authentic standards. Mass spectrum of each target compound was also compared with that of Wiley and NIST mass spectral databases.

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Determination of Antioxidant Activity

The antioxidant capacities of nutmeg oil and its antioxidant components were evaluated by two methods: the DPPH$^\bullet$ free radical scavenging assay and the β-carotene-linoleic acid assay. Standard antioxidants, BHT and α-tocopherol, were used as the controls.

DPPH$^\bullet$ Free Radical Scavenging Assay

Scavenging activities on DPPH$^\bullet$ free radicals by the crude nutmeg oil and its components were determined according to the method of Yamaguchi et al. (*22*) with minor modification. The reaction mixture containing 0.1 mL of sample, 0.3 mL of 0.1 M Tris-HCl (pH 7.4), 0.1 mL of methanol, and 0.5 mL of 0.3 mM DPPH$^\bullet$ was vigorously shaken and incubated in the darkness at room temperature for 10 min. After incubation, the absorbance of the reaction mixture was spectrophotometrically measured at 517 nm and the scavenging activity of DPPH$^\bullet$ free radical was calculated by the following formula:

$$\text{Scavenging activity (\%)} = \left(1 - \frac{\text{absorbance of sample at 517 nm}}{\text{absorbance of control at 517 nm}}\right) \times 100\,\%$$

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β-Carotene–Linoleic Acid Assay

Antioxidant capacities of the crudenutmeg oil and its components were also determined by measuring the β-carotene bleaching (*23*). Three milliliters of β-carotene solution that was prepared by dissolving 5 mg of β-carotene in 50 mL of chloroform was mixed with 50 mg of linoleic acid and 500 mg of Tween 20. The chloroform in the mixture solution was then purged off by high purity nitrogen gas before 100 mL of distilled water was added to the mixture. Sample (0.1 mL) was mixed with 1 mL of the emulsion and incubated at 50°C for 30 min. The reaction mixture without sample was used as a blank. The absorbance of the reaction mixture was spectrophotometrically measured at 470 nm and the antioxidant activity was calculated by the following formula:

$$\text{Antioxidant activity (\%)} = \left(1 - \frac{S_0 - S_{30}}{B_0 - B_{30}}\right) \times 100\%$$

Where S_0 and B_0 are the absorbance of the sample and blank, respectively, before incubation, while S_{30} and B_{30} are the absorbance of the sample and blank, respectively, after incubation at 50°C for 30 min.

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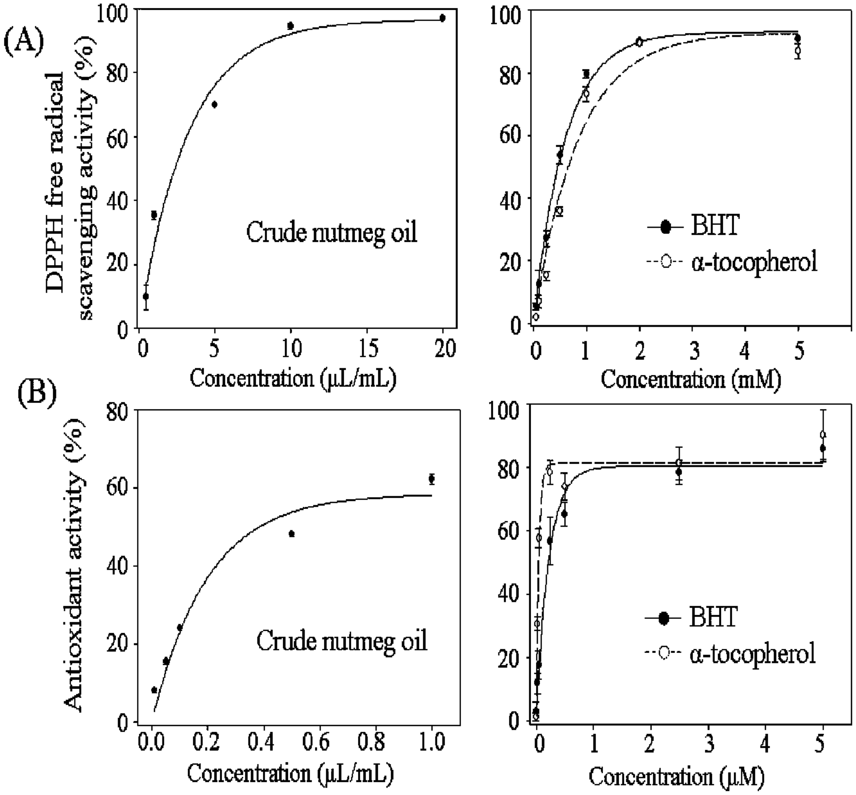

Figure 1. Antioxidant capacities of crude nutmeg oil and antioxidant standard controls were evaluated by: (A) DPPH• free radical scavenging assay; and (B) the β-carotene-linoleic acid assay, which were spectrophotometrically measured at 517 nm and 470 nm, respectively. BHT and α-tocopherol were used as standards.

EC_{50} values that were used to evaluate the antioxidant capacities of the crude oil, identified antioxidant compounds, and standards were the effective concentrations at which DPPH• radicals were scavenged or the β-carotene oxidation was inhibited by 50%.

Statistical Analysis

The data on the antioxidant activities of the crude nutmeg oil and antioxidants identified from the nutmeg oil were subjected to the analysis of variance and analyzed with nonlinear regressions. Statistical analysis was conducted on the SAS V8 software for Windows (SAS Institute Inc., Cary, NC). Differences among all sample means were determined by analysis of variance (AVONA) at $p<0.05$.

Results and Discussion

Antioxidant Capacity of the Crude Nutmeg Oil

Comparison of the antioxidant capacity between the crude nutmeg oil and two common antioxidants (e.g., BHT and α-tocopherol) is shown in Figure 1. In the DPPH$^•$ free radical scavenging assay, the EC_{50} value of the crude nutmeg oil dissolved in methanol was 2.4 μL/mL, at which the scavenging activity was comparable to that of 462 μM BHT and 656 μM α-tocopherol (Figure 1A). By contrast, the inhibitive capacity of the nutmeg oil and its counterparts against the oxidation of β-carotene and linoleic acid was profiled in Figure 1B. After incubation with the β-carotene-linoleic acid emulsion at 50°C for 30 min, the crude nutmeg oil approached its saturated antioxidant activity to approximate 60% with corresponding EC_{50} value at 0.4 μL/mL, while at the same tested condition the EC_{50} values of BHT and α-tocopherol were 43 μM and 9 μM, respectively. These results indicated that the antioxidant capacity of the crude nutmeg oil was quite comparable to that of BHT and α-tocopherol.

Chromatographic Separation of Antioxidants in the Crude Nutmeg Oil

The antioxidants in the crude nutmeg oil were separated by the silica gel open column chromatography and the normal-phase silica-HPLC. One hundred fractions were collected (Figure 2) from the crude nutmeg oil through the silica gel open column, by which the oil was eluted by a sequential gradient of three different solvents, i.e., hexane, DCM, and methanol. Their antioxidant activities were simultaneously determined by the DPPH$^•$ free radical scavenging assay. Four primary antioxidant peaks (I, II, III, and IV) were separated (Figure 2). Peak I was separated by a mixed eluant of hexane/DCM (1:1), Peak II and III by DCM, and Peak IV by a mixture solvent of DCM/methanol (1:1). At the same experimental condition aforementioned, peaks I and IV showed higher DPPH$^•$ free radical scavenging activities at about 95% than that of peak II and III at about 80%.

To simplify further separation of the antioxidants, fractions within the four peaks were respectively pooled into four parts, and concentrated by a rotary evaporator at 40°C. The concentrated four fractions (peaks) were further separated by the Spherisorb silica-HPLC using different linear gradients. Similarly, all collected HPLC fractions were measured by the DPPH$^•$ scavenging activity. As shown in Figure 3, all labeled antioxidants (i.e., A, B, C, D) in each partially purified fractions (I, II, III, and IV) were eluted by less than 50% of DCM/Methanol (1:1) mixture solvent. In addition, with the aid of the on-line DPPH$^•$ free radical scavenging assay each fraction gave only one antioxidant-active peak among the eluted peaks that were separated by HPLC. These antioxidant peaks that were named as A, B, C and D have their radical scavenging activities in 91%, 73%, 71%, and 93%, respectively.

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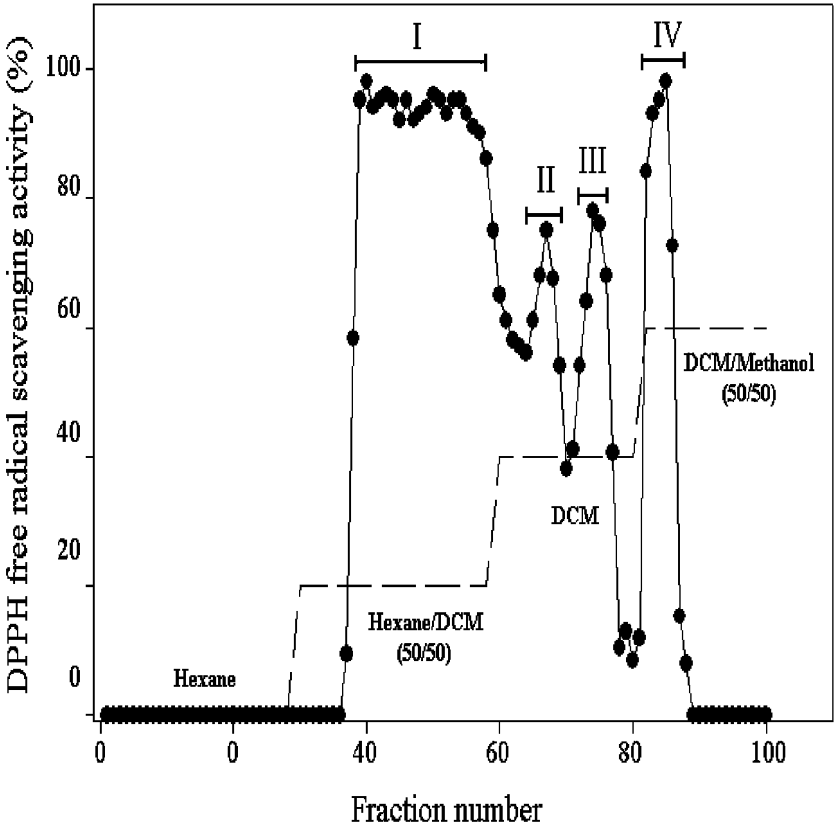

Figure 2. Separation of antioxidants from crude nutmeg oil using silica-gel open column chromatography. The sample was eluted out at a flow rate of 4 mL/min by solvents in the following order: hexane, hexane/DCM (1:1), DCM, and DCM/methanol (1:1). The solid line represents the antioxidant activity of the collected fractions measured by the DPPH$^\bullet$ free radical scavenging assay.

Identification of Antioxidants Partially Separated by Silica-HPLC

Four antioxidant peaks (A, B, C and D) separated by the silica-HPLC were further separated and identified by GC-MS. The results are shown in Figure 4 and Table 1. The crude nutmeg oil had more than 50 compounds including α–pinene (21.3%), sabinene (23.5%), β-pinene (19.1%), terpinene-4-ol (8.7%), and myristicin (9.6%). But these major compounds and other two chemicals (α–terpinolene and γ-terpinene) reported as antioxidants of tea tree oil (*24*) did not exhibit or show strong antioxidant activities in our research using the current two different antioxidant assays. However, three minor compounds (eugenol, isoeugenol, and methoxyeugenol) after chromatographic enrichment were identified and confirmed to be strong antioxidants corresponding to the antioxidant activities of fractions A, B, C and D (Figure 3 and Figure 4). Eugenol, isoeugenol, and methoxyeugenol, which have been reported to possess various bioactivities (*15*, *25–30*), were separated and identified in the peak A, peak B and C, and peak B and D, respectively. Moreover, we did not find other identified compounds (i.e., cis-sabinene hydrate, α–terpineol, and β-terpineol) in the four peaks contributed antioxidant activities to the peaks A-D and the nutmeg oil.

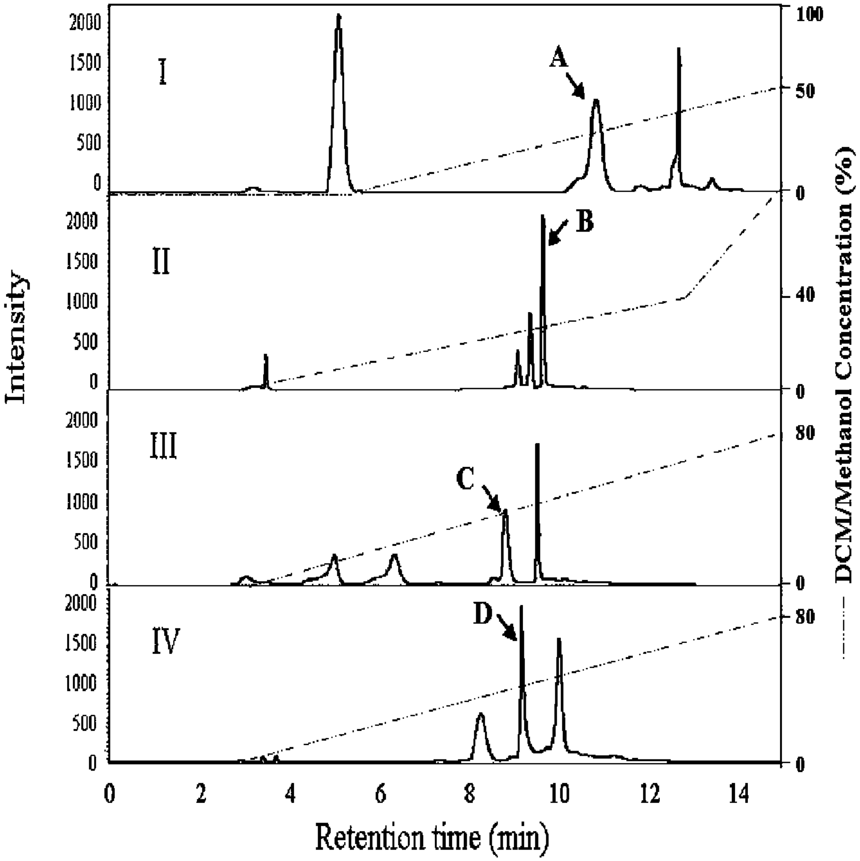

Figure 3. HPLC profiles of the nutmeg antioxidant fractions after being separated by the silica-gel open column. Samples were separated by the Spherisorb silica-HPLC and eluted with a linear gradient of the mixture solvent of DCM/methanol (1:1) at a flow rate of 1 mL/min. The antioxidant peaks labeled in symbols of A, B, C, and D in each fraction (I-IV) were confirmed by the DPPH• free radical scavenging assay. The dotted lines represent the solvent gradient in HPLC analysis.

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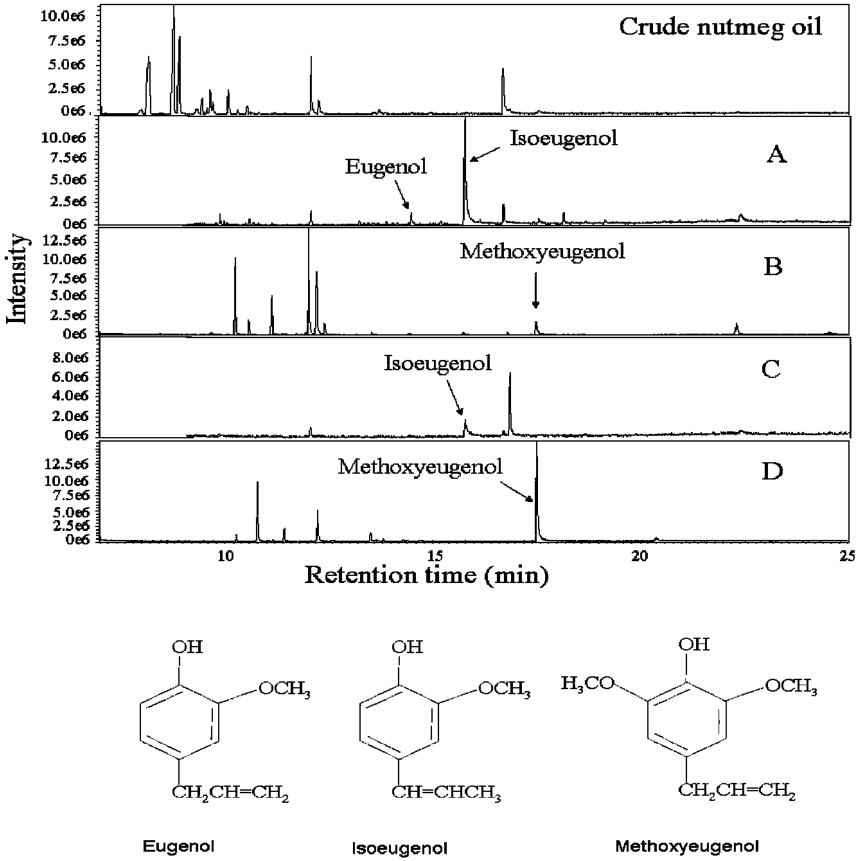

Figure 4. GC TIC profiles of the crude nutmeg oil and antioxidant peaks (A, B, C, and D) separated by HPLC. The chemicals listed are three major antioxidant constituents of nutmeg oil that were identified by GC-MS.

Evaluation of Antioxidant Capacity of Eugenol, Isoeugenol, and Methoxyeugenol Identified from Nutmeg Oil

Antioxidant capacities of three nutmeg constituents, i.e., eugenol, isoeugenol, and methoxyeugenol, were in turn investigated by the aforementioned methods, and compared with BHT and α–tocopherol. As indicated in Table 2, eugenol and methoxyeugenol exhibited stronger DPPH• scavenging activities than the standard counterparts. The EC_{50} values of eugenol and methoxyeugenol were 329 μM and 355 μM, respectively and these values were 30-40% and 85-100% lower than those of BHT (462 μM) and α–tocopherol (656 μM), while the activity of isoeugenol was similar to that of α–tocopherol but weaker than that of BHT. The DPPH• scavenging activities of three antioxidant candidates and two standards were in the following order: eugenol > methoxyeugenol > BHT > isoeugenol >

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α–tocopherol. Unlike the DPPH$^•$ scavenging capacities shown by the compounds, in the β-carotene-linoleic acid assay, the inhibitory activities of three compounds against the oxidation of β-carotene were significantly weaker than the two standard antioxidants (Table 2). Their antioxidant activities were in the following order: α–tocopherol > BHT > isoeugenol > methoxyeugenol > eugenol. The EC_{50} values of isoeugenol (144 μM) and methoxyeugenol (163 μM) were about 3 times lower than that of eugenol (526 μM), but were 3 times and 16-18 times higher than that of BHT (43 μM) and α–tocopherol (9 μM), respectively.

Table 1. Chemical compositions of the crude nutmeg oil and antioxidant peaks (A, B, C, and D) separated by silica-HPLC

Peak no.	*Components*	*RT (min)*	*RI*[a]	*Composition (%)*				
				Crude oil	*Peaks separated by silica-HPLC*			
					A	*B*	*C*	*D*
1	α-thujene	7.98	932	3.5				
2	α-pinene	8.16	942	12.5				
3	sabinene	8.75	981	13.5				
4	β-pinene	8.90	990	12.0				
5	α-terpinene	9.44	1025	4.1				
6	cymene	9.56	1033	1.4				
7	limonene	9.64	1038	6.1				
8	β-phellandrene	9.70	1042	3.5				
9	γ-terpinene	10.07	1065	5.4				
10	cis-sabinene hydrate	10.28	1080	0.9		17.2		26.3
11	α-terpinolene	10.53	1094	2.9				
12	β-terpineol	11.25	1141	>0.1		5.8		5.9
13	terpinene-4-ol	12.06	1194	6.6	5.3	56.1		
14	α-terpineol	12.25	1207	1.3		16.8		17.3
15	safrole	13.73	1309	3.5				
16	eugenol	14.49	1364	0.7	3.9			
17	isoeugenol	15.78	1462	0.9	79.8		30.3	
18	myristicin	16.69	1535	10.6	7.5			

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Table 1. (Continued). Chemical compositions of the crude nutmeg oil and antioxidant peaks (A, B, C, and D) separated by silica-HPLC

Peak no.	*Components*	*RT (min)*	*RI*[a]	*Composition (%)*				
				Crude oil	*Peaks separated by silica-HPLC*			
					A	*B*	*C*	*D*
19	elemicin	16.85	1548	0.8			69.7	
20	methoxyeugenol	17.50	1604	0.4		4.2		50.4

[a] RI was calculated based on the index of a series of *n*-alkanes (C_8-C_{30}).

Table 2. Antioxidant capacities of antioxidants identified from the crude nutmeg oil

	Antioxidant capacity, EC_{50} (µM) *	
	Scavenging activity	*Antioxidant activity*
Eugenol	329^{e}	526^{a}
Isoeugenol	615^{b}	144^{c}
Methoxyeugenol	355^{d}	163^{b}
BHT	462^{c}	43^{d}
α-Tocopherol	656^{a}	9^{e}

* Different superscript symbols in the same column represent significant difference at $P<0.05$.

Conclusions

Although the major constituents of the crude nutmeg oil did not contribute the DPPH• free radical scavenging activity and the inhibitory ability against the oxidation of β-carotene and linoleic acid, eugenol, isoeugenol, and methoxyeugenol were identified and confirmed as the main antioxidant contributors for the nutmeg oil. Those chemicals demonstrated that (1) they had stronger and/or comparable free radicals scavenging activities to BHT and α–tocopherol; (2) they inhibited the peroxidation of linoleic acid and prevented the β-carotene bleaching, though they were weaker antioxidants than the BHT and α–tocopherol in the β-carotene-linoleic acid emulsion system. This result is in agreement with previous research reported by other groups (*27–30*). However, it is also worthy to be noticed that these phenolics, particularly the eugenol, have strong and characteristic flavor of clove, which might make them of less interest for use as antioxidants. Nevertheless, considering the fact that nutmeg powder and nutmeg oil have already been used in the preparation of meat products, soups, sauces, baked foods, confectioneries, puddings, etc., we have strong reasons to

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use nutmeg oil and its antioxidant constituents as useful and functional food ingredients, and to use them in other applications requiring these properties.

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References

1. Burt, S. Essential oils: Their antibacterial properties and potential applications in foods−Review. *Int. J. Food Microbiol.* **2004**, *94*, 223–253.
2. Hammer, K. A.; Carson, C. F.; Riley, T. V. Antimicrobial activity of essential oils and other plant extracts. *J. Appl. Microbiol.* **1999**, *86*, 985–990.
3. Jayaprakasha, G. K.; Jena, B. S.; Negi, P. S.; Sakariah, K. K. Evaluation of antioxidant activities and antimutagenicity of turmeric oil: A byproduct from curcumin production. *Zeitschrift für Naturforschung C.* **2002**, *57C*, 828–835.
4. Leal, P. E.; Braga, M. E.; Sato, D. N.; Carvalho, J. E.; Marques, M. O. M.; Meireles, M. A. A. Functional properties of spice extracts obtained via supercritical fluid extraction. *J. Agric. Food Chem.* **2003**, *51*, 2520–2525.
5. Carnesecchi, S.; Langley, K.; Exinger, F.; Gosse, F.; Raul, F. Geraniol, a component of plant essential oils, sensitizes human colonic cancer cells to 5-fluorouracil treatment. *J. Pharmacol. Exp. Ther.* **2002**, *301* (2), 625–630.
6. Courreges, F.; Courreges, M. C. In vitro and in vivo activity of eugenol on human herpesvirus. *Phytother. Res.* **2000**, *14*, 495–500.
7. Ehlers, D.; Kirchhoff, J.; Gerard, D.; Quirin, K. W. High-performance liquid chromatography analysis of nutmeg and mace oils produced by supercritical CO_2 extraction, comparison with steam-distilled oils, comparison of East Indian, West Indian and Papuan oils. *Int. J. Food Sci. Technol.* **1998**, *33*, 215–223.
8. Morita, T.; Jinno, K.; Kawagishi, H.; Arimoto, Y.; Suganuma, H.; Inakuma, T.; Sugiyama, K. Hepatoprotective effect of myristicin from nutmeg (*Myristica fragrans*) on lipopolysaccaride/D-galactosammine-induced liver injury. *J. Agric. Food Chem.* **2003**, *51*, 1560–1565.
9. Ram, A.; Lauria, P.; Gupta, R.; Sharma, V. N. Hypolipidaemic effect of *Myristica fragrans* fruit extract in rabbits. *J. Ethnopharmacol.* **1996**, *55*, 49–53.
10. Choo, L. C.; Wong, S. M.; Liew, K. Y. Essential oil of nutmeg pericarp. *J. Sci. Food Agric.* **1999**, *79*, 1954–1957.

11. Spricigo, C. B.; Pinto, L. T.; Bolzan, A.; Novais, A. F. Extraction of essential oil and lipids from nutmeg by liquid carbon dioxide. *J. Supercrit. Fluids* **1999**, *15*, 253–259.
12. Sangalli, B. C.; Chiang, W. Toxicology of nutmeg abuse. *Clin. Toxicol.* **2000**, *38*, 671–678.
13. Stein, U.; Greyer, H.; Hentschel, H. Nutmeg (myristicin) poisoning: Report on a fatal case and a series of cases recorded by a poison information centre. *Forensic Sci. Int.* **2001**, *118*, 87–90.
14. Van Gils, C; Cox, P. A. Ethnobotany of nutmeg in the Spice Islands. *J. Ethnopharmacol.* **1994**, *42*, 117–124.
15. Janssens, J.; Laekeman, G. M.; Pieters, A. C.; Totte, J.; Herman, A. G.; Vlietinck, A. J. Nutmeg oil: Identification and quantitation of its most active constituents as inhibitors of platelet aggregation. *J. Ethnopharmacol.* **1990**, *29*, 179–188.
16. Takikawa, A.; Abe, K.; Yamamoto, M.; Ishimaru, S.; Yasui, M.; Okubo, Y.; Yokoigawa, K. Antimicrobial activity of nutmeg against *Escherichia coli* 0157. *J. Biosci. Bioengin.* **2002**, *94*, 315–320.
17. Valero, M.; Salmerón, M. C. Antibacterial activity of 11 essential oils against *Bacillus cereus* in tyndallized carrot broth. *Int. J. Food Microbiol.* **2003**, *85*, 73–81.
18. Dorman, H. J. D.; Figueiredo, A. C.; Barroso, J. G.; Deans, S. G. *In vitro* evaluation of antioxidant activity of essential oils and their components. *Flavour Fragr. J.* **2000**, *15*, 12–16.
19. Calucci, L.; Pinzino, C.; Zandomeneghi, M.; Capocchi, A.; Ghiringhelli, S.; Saviozzi, F.; Tozzi, S.; Galleschi, L. Effects of γ-irradiation on the free radical and antioxidant contents in nine aromatic herbs and spices. *J. Agric. Food Chem.* **2003**, *51*, 927–934.
20. Murcia, M. A.; Egea, I.; Romojaro, F.; Parras, P.; Jiménez, A. M.; Martínez-Tomé, M. Antioxidant evaluation in dessert spices compared with common food additives. Influence of irradiation procedure. *J. Agric. Food Chem.* **2004**, *52*, 1872–1881.
21. Tomaino, A.; Cimino, F.; Zimbalatti, V.; Venuti, V.; Sulfaro, V.; De Pasquale, A.; Saija, A. Influence of heating on antioxidant activity and the chemical composition of some spice essential oils. *Food Chem.* **2005**, *89*, 549–554.
22. Yamaguchi, T.; Takamura, H.; Matoba, T.; Terao, J. HPLC method for evaluation of the free radical-scavenging activity of foods by using 1,1-diphenyl-2-picrylhydrazyl. *Biosci. Biotechnol. Biochem.* **1998**, *6*, 1201–1204.
23. Kumazawa, S.; Taniguchi, M.; Suzuki, Y.; Shimura, M.; Kwon, M. S.; Nakayama, T. Antioxidant activity of polyphenols in carob pods. *J. Agric. Food Chem.* **2002**, *50*, 373–377.
24. Kim, H. J.; Chen, F.; Wu, G.; Wang, X.; Chung, H. Y.; Jin, Z. Evaluation of antioxidant activity of Australian tea tree (*Melaleuca alternifolia*) oil and its components. *J. Agric. Food Chem.* **2004**, *52*, 2849–2854.
25. Munerato, M. C.; Sinigaglia, M.; Reguly, M. L.; de Andrade, H. H. R. Genotoxic effects of eugenol, isoeugenol and safrole in the wing spot test of

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Drosophila melanogaster. *Genet. Toxicol. Environ. Mutagen.* **2005**, *582*, 87–94.

26. Rompelberg, C. J. M.; Ploemen, J. H.; Jespersen, S.; van der Greef, J.; Verhagen, H.; van Bladeren, P. J. Inhibition of rat, mouse, and human glutathione S-transferase by eugenol and its oxidation products. *Chem. Biol. Interact.* **1996**, *99*, 85–97.
27. Abraham, S. K. Anti-genotoxicity of trans-anethole and eugenol in mice. *Food Chem. Toxicol.* **2001**, *39*, 493–498.
28. Ito, M.; Murakami, K.; Yoshino, M. Antioxidant action of eugenol compounds: Role of metal ion in the inhibition of lipid peroxidation. *Food Chem. Toxicol.* **2005**, *43*, 461–466.
29. Kumaravelu, P.; Subramaniyam, S.; Dakshinamoorthy, D. P.; Devaraj, N. S. The antioxidant effect of eugenol on CCl_4-induced erythrocyte damage in rats. *Nutr. Biochem.* **1996**, *7*, 23–28.
30. Rauscher, F. M.; Sanders, R. A; Watkins, III, J. B. Effects of isoeugenol on oxidative stress pathways in normal and streptozotocin-induced diabetic rats. *J. Biochem. Mol. Toxicol.* **2001**, *15*, 159–164.

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Chapter 16

Characterization of Acylated Flavonoid Glycosides from Sea Buckthorn (*Hippophaë rhamnoides*) Juice Concentrate by Preparative HSCCC/ESI-MS-MS

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A direct coupling of preparative high-speed countercurrent chromatography to electrospray ionization mass spectrometry (HSCCC/ESI-MS-MS) was used for a '*target-guided*' isolation and characterization of flavonoids with novel malic acid ester functionalities detected in sea buckthorn juice concentrate (*Hippophaë rhamnoides* L. ssp. *rhamnoides*, Elaeagnaceae). Formation of flavonoid malyl-ester derivatives is induced under high acidic processing conditions and elevated thermal impact. In general, the berries of *Hippophaë rhamnoides* are well known to be a rich source for flavonoids, carotenoids, vitamins, and lipids and are traditionally used in the ethnopharmacy of Tibet, Mongolia, China, and Central Asia. Many health claims are associated with sea buckthorn. The hyphenation experimental study of preparative HSCCC and ESI-MS/MS provided novel and immediate structural insights to an altered flavonoid profile of a commercial sea buckthorn juice concentrate.

Sea buckthorn berries are known to be an excellent source for flavonoids, carotenoids, vitamins, and lipids. Therefore, the overall nutritional values and health related effects were well documented (*1*–*5*). This investigation covers the application of preparative high-speed countercurrent chromatography (HSCCC) as a unique all liquid-liquid separation technique in direct combination with

In Flavor and Health Benefits of Small Fruits; Qian, M., et al.;
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sensitive electrospray mass spectrometry detection and monitored a modified polyphenol profile in sea buckthorn juice concentrate (*Hippophaë rhamnoides* L. ssp. *rhamnoides*, Elaeagnaceae).

Counter-current chromatography (CCC) in general uses the distribution effects of analytes between two immiscible liquid phases and elution of compounds is strictly dependent on polarities. Chemisorptive effects of polar natural products documented for solid phase materials, which hampers the recovery of polyphenols such as flavonoids can be completely omitted by CCC, and thermally labile, and light-sensitive bioactive natural products can be effectively isolated (*6*, *7*). In case of detection, mass-spectrometry is still the most sensitive technique and also the best choice for gaining rapid structural data on components in natural product crude extracts. Direct on-line connections of high-speed countercurrent chromatography have been developed for various analytical applications (HSACCC/ mass-spectrometry) and were operated with differing ionization techniques (*8–11*). Preparative scale high-speed countercurrent chromatography connected to electrospray ionization mass spectrometry (HSCCC/ESI-MS/MS) was firstly realized for sea buckthorn polyphenols (*12*).

Preparative CCC with ESI-MS/MS detection using selected single ion-traces (Fig. 1) can visualize strongly overlaid and co-eluting HSCCC-peaks. Substances of potential interest can still be recognized in complex matrices (*target-guided-screening*). Therefore, this methodology is a new valuable tool in the field of preparative natural product isolation.

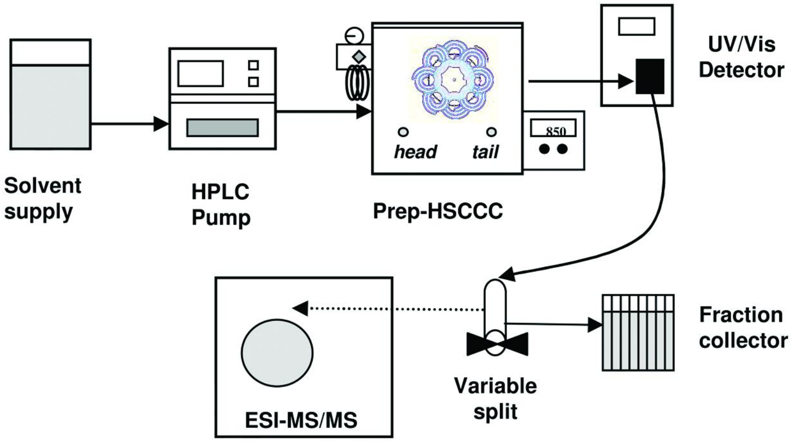

Figure 1. Schematic diagram of the preparative HSCCC instrument (multilayer coil planet centrifuge model CCC 1000, Pharma-Tech Research Corp., U.S.A.) coupled to a Bruker Esquire LC-ESI-MS/MS ion trap multiple mass-spectrometer (Bruker Daltonics, Bremen, Germany) in negative ionization mode analyzing ions from m/z 50 up to m/z 1500 amu.

In this study, the generated continuous molecular weight data provided complete MS/MS fragmentation pattern (Fig. 2, Fig. 3) and led to the recognition of novel malic acid ester derivatives which occurred in the investigated crude solvent partition of *Hippophaë rhamnoides* berry juice concentrate.

Most of the detected novel structures were tentatively assigned (cf. Fig. 3). ESI-MS/MS structural informations from fragmentation pattern is limited and the exact positions of acylations in the flavonoid glycosides cannot be assigned. Also the definition which of the two carboxyl groups in the malic acid moiety had reacted to from the ester function cannot be deduced from the MS/MS fragmention pathways.

Flavonoid glycoside malyl-ester formation in juice concentrates was still unknown and biological activity of these components could be of interest.

In addition acyl-linked flavonoid glycosides could be further evaluated as specific marker components to distinguish directly pressed homestyle made fruit juices from reconstituted concentrated products and pure reference compounds are needed and could be obtained by preparative HSCCC in combination with a final sample clean-up by C18-HPLC.

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Experimental Procedure

Concentrate Processing

The sea buckthorn fruits used for the study were harvested in Romania in September 2005. The frozen berries were preheated before mashing. The mash was subjected to a treatment with pectolytic enzymes (1 – 2h, 52 °C) and separated into juice and pomace by a decanter machine. The turbid juice product, highly concentrated in pulp and oil was clarified by a plate separator. The resulting sea buckthorn juice was clarified using fining agents, such as bentonite (8–12 h, 10–12 °C). After filtration under vacuum with diatomeous earth, the clear juice was concentrated by thermovacuum evaporation (five stage evaporator, 80–85 °C). The °Brix value was 65 for clear juice concentrates. Before aseptic filling the juice was treated by a HTST process (high-temperature-short-time treatment: 90°C, 45 s) and rechilled, immediately.

Experimental of HSCCC-ESI-MS-MS

The preparative HSCCC instrument used in the present study was a multilayer coil planet centrifuge model CCC 1000 (Pharma-Tech Research Corp., U.S.A.), equipped with three preparative coils connected in series (polytetrafluorethylene (PTFE) tubing: 2.6 mm i.d. x 165 m, 850 mL total volume). A manual sample injection valve with a 25 mL loop was used to introduce the sample into the coil system. The mobile phase was delivered with a Biotronik BT 3020 HPLC pump (Jasco, Grossumstadt, Germany).

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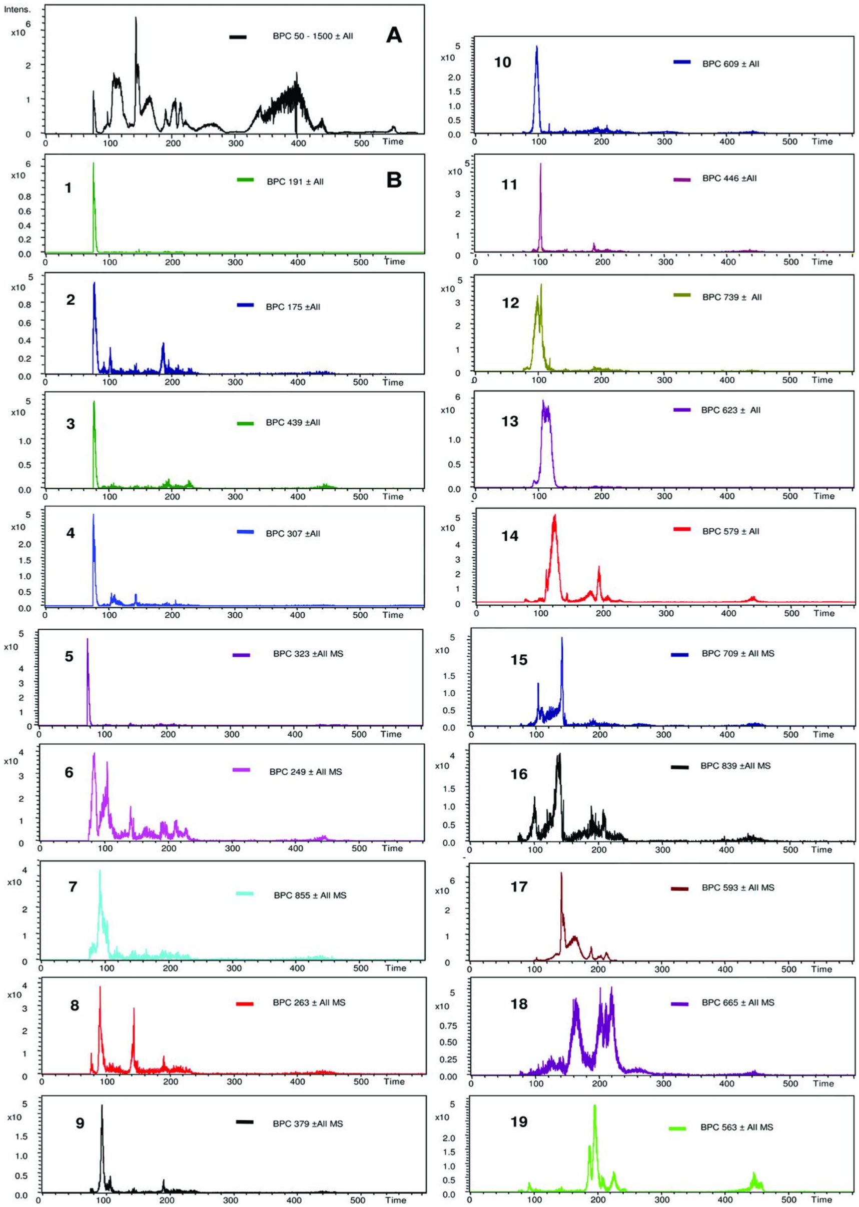

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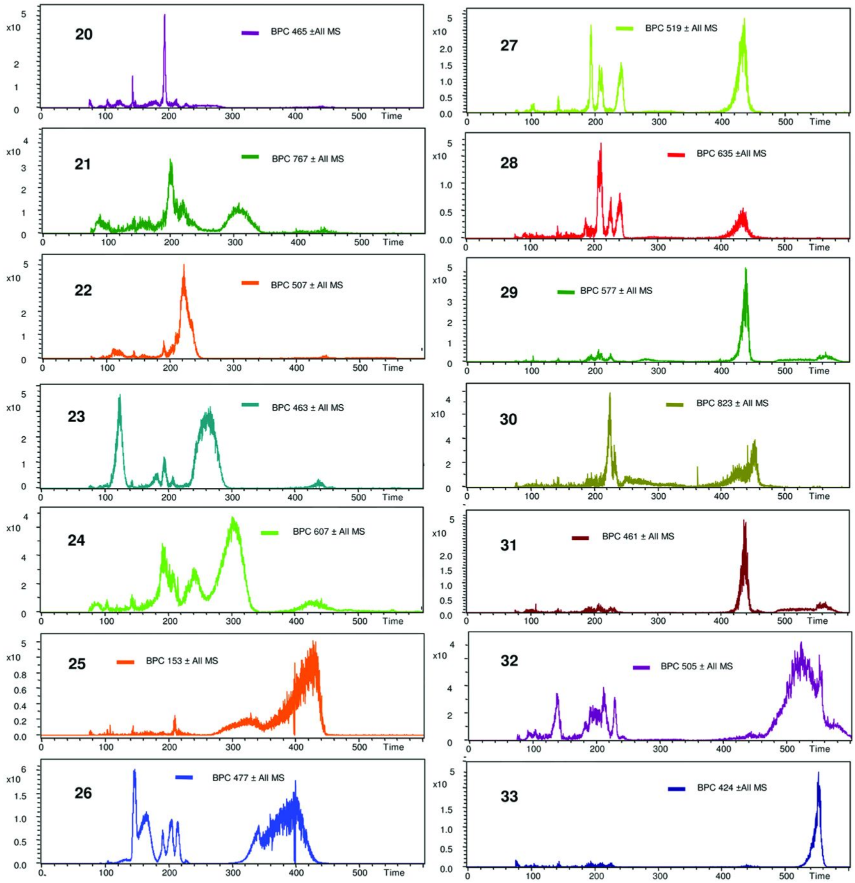

Figure 2. Preparative HSCCC-ESI-MS chromatogram.
A: total ion ESI-MS trace (neg. ionization mode) 50-1500 Da.
B: selected ESI-MS single ion traces of flavonoid-glycosides partially assorted by increasing CCC-retention times.
HSCCC conditions: flow rate: 3.0 mL/min;
CCC-operation: 'head-to-tail mode'; velocity: 850 rpm.
Ethyl acetate crude partition of sea buckthorn berry juice concentrate separated with the biphasic solvent system n-hexane - n-butanol - water (1:1:2, v/v/v).
Injection amount 500 mg.

A injection of 500 mg of the polyphenol-enriched ethylactetate partition from the sea buckthorn juice concentrate was used for the hyphenation experiment using the biphasic solvent system consiting of *n*-hexane - *n*-butanol - water (1:1:2, *v/v/v*).

A schematic diagram of the HSCCC/ESI-MS-MS hyphenation system is presented in Figure 1. The preparative HSCCC-separation was operated at a flow rate of 3.0 mL/ min, and rotation velocity of the main centrifuge coil rotor was set to 850 rpm. After passing through a UV/Vis detector monitored at λ 280 nm (chromatogram not presented here), the eluate was directed to a variable

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low pressure micro-splitter valve (UpChurch Scientific®, Oak Harbour, WA, U.S.A.) where a split ratio of 1 : 125 was adjusted. This resulted in a small incoming flow rate of 24 µL/ min to the ESI-MS/MS interface to prevent any risk of contamination in the ion source compartment. The main effluent stream was guided into test tubes to recovery the separated compounds using a preparative fraction collector (LKB SuperRac 2211, LKB, Bromma, Sweden) at 4.0 min intervals per tube.

The distance between the variable split unit to the ESI-MS/MS was kept short with a metal HPLC capillary (l: 3 cm, i.d.: 0.17 mm). All used PTFE tubings for connecting the HSCCC with the UV-detector, T-unit, variable splitter and the ESI-MS/MS had an internal diameter of 1.0 mm.

The continuous acquisition of mass-spectrometry data by ESI-MS-MS during the HSCCC-experiments was performed on a Bruker Esquire LC-MS ion trap multiple mass-spectrometer (Bruker Daltonics, Bremen, Germany) in negative ionization mode analyzing ions from *m/z* 50 up to *m/z* 1500 amu.

Drying gas was nitrogen (flow 5.0 L min^{-1}, 310 °C), and nebulizer pressure was set to 10 psi. ESI-MS-MS parameters (neg. mode): capillary +3500 V, end plate +3000 V, capillary exit -90 V, capillary exit offset -60 V, skim 1 -30 V, skim 2 -10 V; trap drive 53.0, target mass range *m/z* 500 amu, compound stability 100%, trap drive level 120%, ICC target 30000, MS/MS experiments afforded a fragmentation amplitude value of 1.2 V.

Volumetric measurement showed a retention of 90% of stationary phase solvent. The applied biphasic solvent system was stable and no '*carry-over*' of stationary phase was observed. Stationary phase retention is one of the most critical aspects for performing successful hyphenated HSCCC/ESI-MS-MS analysis to obtain stable ESI-MS signals.

Results and Discussion

Structure Elucidation of Sea Buckthorn Flavonoids by ESI-MS-MS Fragmentation

For the direct ESI-MS/MS measurement of the eluting components from the preparative CCC coil-system, the negative ionization mode was applied for the best sensitivity to detect polyphenol structures. All mass-spectrometrical data for the presented structural assignments, such as molecular weights ($[M-H]^-$) and selected ESI-MS-MS fragment ion traces were measured on-line (cf. Fig. 2). Isorhamnetin flavonoid glycosides can be regarded as typical marker components which principally are present in all sea buckthorn berry products such as concentrates, juices, and jams.

From recent literature, a rather large amount of flavonoid glycosides is already documented (*2*, *13–15*). Interestingly not all of these were recognized during the HSCCC-ESI-MS/MS experiment, but nevertheless the malic acid substituted derivatives – possibly unique for the juice concentrate products - were detected as novel natural products.

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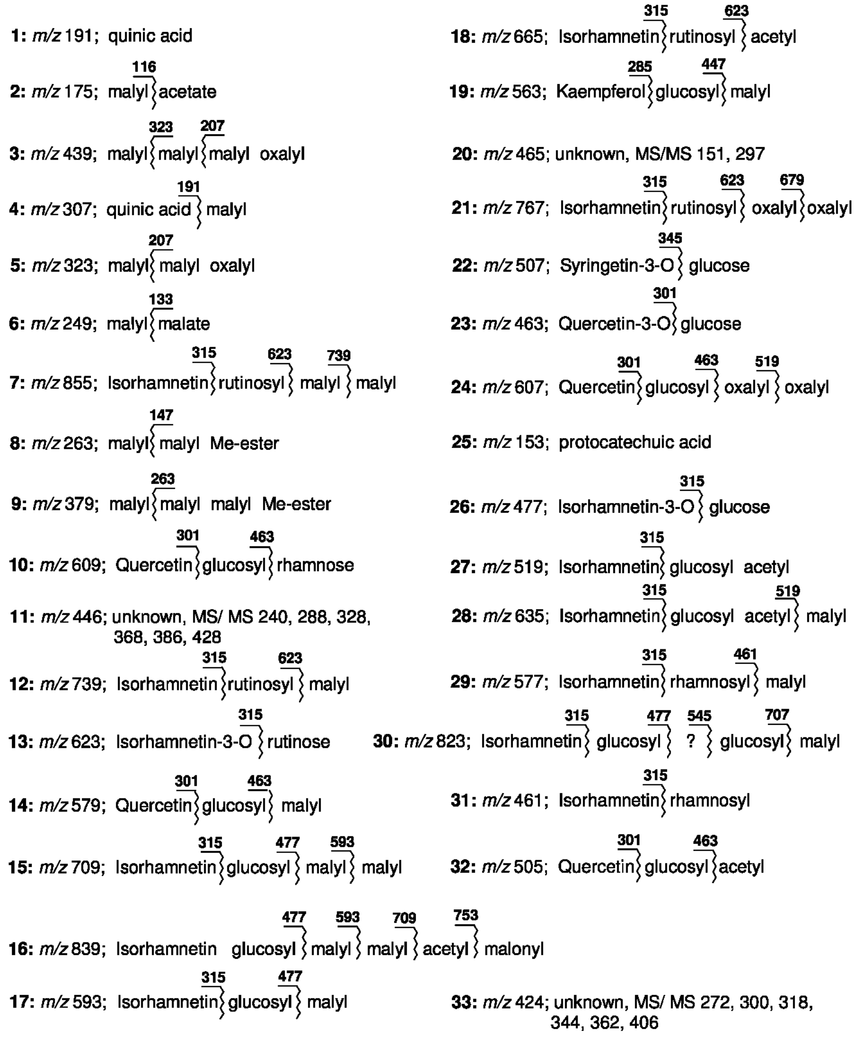
1: m/z 191; quinic acid
2: m/z 175; malyl 116 acetate
3: m/z 439; malyl 323 malyl 207 malyl oxalyl
4: m/z 307; quinic acid 191 malyl
5: m/z 323; malyl 207 malyl oxalyl
6: m/z 249; malyl 133 malate
7: m/z 855; Isorhamnetin 315 rutinosyl 623 malyl 739 malyl
8: m/z 263; malyl 147 malyl Me-ester
9: m/z 379; malyl 263 malyl malyl Me-ester
10: m/z 609; Quercetin 301 glucosyl 463 rhamnose
11: m/z 446; unknown, MS/ MS 240, 288, 328, 368, 386, 428
12: m/z 739; Isorhamnetin 315 rutinosyl 623 malyl
13: m/z 623; Isorhamnetin-3-O 315 rutinose
14: m/z 579; Quercetin 301 glucosyl 463 malyl
15: m/z 709; Isorhamnetin 315 glucosyl 477 malyl 593 malyl
16: m/z 839; Isorhamnetin glucosyl 477 malyl 593 malyl 709 acetyl 753 malonyl
17: m/z 593; Isorhamnetin 315 glucosyl 477 malyl
18: m/z 665; Isorhamnetin 315 rutinosyl 623 acetyl
19: m/z 563; Kaempferol 285 glucosyl 447 malyl
20: m/z 465; unknown, MS/MS 151, 297
21: m/z 767; Isorhamnetin 315 rutinosyl 623 oxalyl 679 oxalyl
22: m/z 507; Syringetin-3-O 345 glucose
23: m/z 463; Quercetin-3-O 301 glucose
24: m/z 607; Quercetin 301 glucosyl 463 oxalyl 519 oxalyl
25: m/z 153; protocatechuic acid
26: m/z 477; Isorhamnetin-3-O 315 glucose
27: m/z 519; Isorhamnetin 315 glucosyl acetyl
28: m/z 635; Isorhamnetin 315 glucosyl acetyl 519 malyl
29: m/z 577; Isorhamnetin 315 rhamnosyl 461 malyl
30: m/z 823; Isorhamnetin 315 glucosyl 477 ? 545 glucosyl 707 malyl
31: m/z 461; Isorhamnetin 315 rhamnosyl
32: m/z 505; Quercetin 301 glucosyl 463 acetyl
33: m/z 424; unknown, MS/ MS 272, 300, 318, 344, 362, 406

*Figure 3. Identified and tentatively assigned structures **1** – **33** by direct coupling of preparative HSCCC-ESI-MS/MS.*

The identified (some tentatively identified) components from sea buckthorn juice concentrate were listed according to the retention times (Rt) of the HSCCC-separation (component list cf. Fig. 3). In the general operation mode of the CCC equipment, the so-called '*head-to-tail*' direction, the most polar components tend to elute early and less polar components later, due to a stronger affinity to the liquid and more organic stationary phase (*6*).

Most of the ESI-MS/MS detected compounds revealed a malic acid substitution. This was clearly observed by a neutral loss of Δ*m*/*z* 116 (malic acid Mr: 134 amu). Nevertheless, the observed MS/MS cleavages were not able to indicate the exact positions of such esterifications, only the existence of malic acid(s) as partial structural element in the molecule are valid.

Very polar malic acid oligomers were also detected (cf. Fig. 2 and Fig. 3 , **1** - **9**): reaction possibilities of malic acid to small oligmers (dimers, trimers) include a large possibility of variation in the reactions. Each malic acid momomer - a non-symmetric organic acid - is principally eligible to react due to its trifunctionality based on a dicarboxylic nature and a single hydroxyl-group in the esterification process. The strong tendency of polymerization due to multiple esterifications under high acidic media is most likely.

During the preparative HSCCC/ESI-MS-MS coupling experiment a highly polar 'window' with extremely polar substances (75 - 120 min) was obvious, with quite a lot of components (**1** - **15**) being co-eluted, nevertheless informative MS/MS fragment data were successfully extracted from the selected ion-traces. The following substances could be detected:

Quinic acid (**1**) with *m/z* 191 was eluted as a sharp peak and was one of the most polar components (Rt ~76 min).

For compound **2**, we postulated a structure of malic acid-acetate. It had an ion signal $[M-H]^-$ at *m/z* 175 and also a clear MS/MS fragment-ion at *m/z* 116.

Compound **3** had an ion signal at *m/z* 439, and showed neutral cleavages of two malyl-functions (Δ*m/z* 116) with respective fragment ions at *m/z* 323 and *m/z* 207 (malyl-oxalyl). We suggested an esterified trimer of malic acid with oxalic acid substitution (Rt ~ 78 min).

Related to **1**, a malyl-ester (**4**) was recognized with $[M-H]^-$ at *m/z* 307, and the fragmentation to a quinic acid moiety at *m/z* 191 (Rt ~78 min). The position of esterification remained unclear.

Component **5** gave a single peak with *m/z* 323. This molecular peak with MS/MS fragmentation of malyl-moiety to *m/z* 207 was clearly related to the partial structure (malyl-oxalyl) observed for compound **3**, and we deduced a dimeric ester of two molecules malic acid with an additional oxalic acid substitution in **5**.

For the compound at *m/z* 249 (**6**), the ion-signal was detected at Rt ~ 84 min and a fragment ion at *m/z* 133 related to a malic acid dimerization product was observed. The later eluting peaks with the same molecular weights were not presenting MS/MS signals, so no more detailed structural informations resulted.

Compound **7** with the ion signal $[M-H]^-$ at *m/z* 855 (Rt ~ 93 min) was showing the cleavage of two malyl-residues (*m/z* 739 and m/z 623) and one rutinose unit leading to the flavonoid aglycone isorhamnetin with *m/z* 315 in the MS/MS fragmentation.

The selected ion trace of *m/z* 263 (**8**) was related to the methyl-ester of the already observed malic acid dimer (**6**) with the respective fragment ion at *m/z* 147. The later eluting peak with the same molecular was not giving any clear MS/MS data (cf. Fig. 2).

For compound **9** with $[M-H]^-$ at *m/z* 379, we postulated a malic acid trimer with an additional methyl-ester function. This will be corroborated by the MS/MS cleavage resulting in *m/z* 263 which was the molecular ion peak of the malic acid dimer methyl ester (**8**).

Compound **10** with $[M-H]^-$ at *m/z* 609 exhibited a quercetin moiety (*m/z* 301). Further MS/MS fragmentation suggested a glucose and rhamnose substitution.

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The identification of compound **11** with an even pseudomolecular ion peak [M-H]$^-$ at *m/z* 446 and the resulting fragment ions with *m/z* at 428, 386, 368, 328, 288, 240 remained unclear - but are potentially related to an alkaloid based structure with an uneven number of nitrogen atoms (also cf. **33**).

Based on the molecular ion peak [M-H]$^-$ at *m/z* 739, and the characteristic MS/MS fragment ions, we postulated an isorhamnetin backbone for the flavonoid **12** by observation of a malic acid cleavage (*m/z* 623) and a rutinosyl-moiety (*m/z* 315).

The molecular ion peak [M-H]$^-$ of **13** at *m/z* 623 (Rt 110 min) was a principal component in the sea buckthorn concentrate (cf. Fig. 2) and was elucidated by 2D-NMR analysis to be isorhamnetin-3-O-rutinose (*15*).

Flavonoid glycoside malic acid derivatives (cf. Fig. 3): The ion trace for *m/z* 579 (**14**) was showing one intense ion signal at Rt ~125 min and two minor intense peak signals. All three showed identical MS/MS fragments at *m/z* 301, 463 suggesting they were related to a quercetin-glucoside with a single malic acid substitution. Different substitution pattern of the sugar and malic acid are obviously causing different retention times during the HSCCC-separation.

Two peaks were detected in the selected ion trace of *m/z* 709 (**15**) - but solely the last one gave the indicative fragment ions at *m/z* 593, 477, 315, suggesting the cleavage of two malic acids (Δ*m/z* 116) and one glucose unit from the isorhamnetin backbone.

The selected ion trace at *m/z* 839 of the HSCCC-ESI-MS coupling experiment detected two higher molecular weight acyl-flavonoid glycosides, and only component **16** (Rt ~135 min) gave characteristic fragement ions (*m/z* 753, 709, 593, 477). The cleavages of one malonyl, then one actetate and lately two malyl-functions to a isorhamnetin-3-glucoside were indicated.

The selected ion trace at *m/z* 593 (**17**) detected three components (Rt ~153 min, ~162 min, ~188 min) with identical ESI-MS/MS fragmentation pattern presenting a malyl- (*m/z* 477) and hexose- (glucose) cleavage to isorhamnetin (*m/z* 315). Changes in malic acid substitution result in positional isomers with varied polarities which could potentially influence the chromatographic behaviour during the HSCCC separation.

The selected ion trace of [M-H]$^-$ at *m/z* 665 detected four different compounds with the same molecular weight. Soley, the last peak **18** showed the necessary MS/MS fragment ions (*m/z* 623, 315) as indication of an acetyl substitution (Δ*m/z* 42), a loss of a rutinosyl-moiety (*m/z* 315) to the isorhamnetin backbone. In theory there are many possibilities to vary the acylation positions in the diglycoside unit. The acetyl-group significantly enlarged the lipophilicity of **18** in respect to the genuine non-acylated compound **13** (*m/z* 623).

In case of the large peak at Rt ~197 min, we postulated a malyl-ester derivative of kaempferol-glucoside (**19**) with [M-H]$^-$ at *m/z* 563. The fragment ion at *m/z* 447 showed the typical neutral loss of malic acid (Δ*m/z* 116) to the agylcone at *m/z* 285.

The ion signal for compound **20** [M-H]$^-$ was detected at *m/z* 465. So far the observed MS/MS fragment ions at *m/z* 151, and 297 remained unclear and were not sufficient for a structural proposal.

The selected ion trace at *m/z* 767 showed two larger peaks (Rt ~201, 308 min) with decent retention time differences. Solely, the later eluting peak 21 gave

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a significant ESI-MS/MS fragmentation and led to isorhamnetin-rutinose with a di-oxalyl substitution (characteristic fragment ions: *m/z* 749/ 750, 721, 679, 651, 623, and 315).

As the principal component of the sea buckthorn berry juice concentrate syringetin-3-O-glucoside (**22**) was identified with $[M-H]^-$ at *m/z* 507 as a single peak of high ion abundance.

For the ion trace $[M-H]^-$ at *m/z* 463 (**23**) at Rt ~467 min, we elucidated its structure as quercetin-3-O-glucoside. The other observed earlier eluting ion peaks were related to the various malyl-flavonoid glycosides **14** showing an immediate cleavage of malic acid in the direct MS-ion trace.

Similar to compound **21** we saw at $[M-H]^-$ at *m/z* 607 and characteristic MS/MS fragment ions at *m/z* 589, 561, 519, 463, 301 for compound **24** suggesting a di-oxalyl substituted quercetin-glucoside.

The very polar and small size polyphenol protocatechuic acid (**25**) eluted late as a broadened peak from the HSCCC coil-system and gave a $[M-H]^-$ signal at *m/z* 153. The strong retention time shift is related to hydrogen bonding and dimerization of acids in highly concentrated solutions (*12*). The ion abundance of fragment ion at *m/z* 109 generated by decarboxylation was much higher than the parent ion.

Isorhamnetin-3-O-glucoside (**26**) as one of the principal flavonoid glycosides in sea buckthorn berry concentrate had a rather enlarged elution time over ~110 min, and gave an intense ion signal at *m/z* 477 during the HSCCC-ESI-MS/MS coupling experiment. The detected peaks with an identical molecular weight, but shorter elution times were directly formed fragment ions. They belonged to the parent ions of *m/z* 593 (**17**), easily generated by the loss of their malyl-functions.

The extracted single ion trace *m/z* 519 led to five different peaks with $[M-H]^-$. All of them showed a clear MS/MS fragmentation to an isorhamnetin backbone. From the MS data we postulated the existence of different acetylated isorhamnetin-glucosides (**27**). Interestingly, depending on the retention time the most abundant component appeared to be more lipophil than the others and eluted at retention time of ~433 min.

For the ion trace at *m/z* 635 four components were detected and MS/MS fragment ions led to identification of isorhamnetin-glucosides (**28**) with one malate and a potential acetate substitution (*m/z* 42). Due to different intensities of observed fragment ions and varying retention times the existence of positional isomers was concluded.

Compound **29** was eluted as a single peak and ESI-MS/MS data with $[M-H]^-$ at *m/z* 577 and clear fragment ions at *m/z* 461 and 315 led to the proposal of an isorhamnetin-rhamnoside with malic acid substitution.

Two larger molecular weight components (**30**) were detected at *m/z* 823 - but with extremely different elution times (Rt ~225 and ~453 min). The extracted ESI-MS/MS fragmentation data were almost identical and suggested isorhamnetin-di-glucosides with one malyl-substitution ($\Delta m/z$ 116).

The isorhamnetin-rhamnoside (**31**) with $[M-H]^-$ was detected at *m/z* 461 with MS/MS at *m/z* 315.

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Compound **32** with [M-H]$^-$ at *m/z* 505 could be an acetyl-derivative of quercetin-glucoside (**23**) as indicated by a neutral loss of Δ*m/z* 42 in the MS/MS ion-trace.

Similar to compound **11**, we also suggested an alkaloid based lipophilic component for **33** with the even pseudomolecular ion peak [M-H]$^-$ at *m/z* 424. So far the strong fragment ions at *m/z* with 406, 362, 344, 318, 300, 272 suggested an uneven number of nitrogen atoms.

Genuine flavonoid glycosides of sea buckthorn berries such as **13**, **22**, **23**, **25**, and **26** were purified and structures were unambiguously elucidated by 1D-, and 2D-NMR experiments . Beside the malyl-and organic acid esterified flavonoid glycosides, thermally induced during the juice concentrate process, syringetin-3-O-glucoside (**22**) was found which was so far not known for plant materials of sea buckthorn (*Hippophaë rhamnoides*).

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Formation of Malic Acid Esters of *Hippophaë* flavonoid Glycosides

During juice concentrate processing, the material was treated with elevated temperatures and vacuum to remove water. Under these conditions, the formation of flavonoid glycoside esters is accelerated in the presence of highly concentrated organic acids such as malic acid in sea buckthorn juice (1.9 - 3.6 g /100 g) (*14*). The chemical balance will be moved to the esterified flavonoid product side by evaporation of water and input of activation energy. So it is quite obvious that a high content of malic acid in any juice material is beneficial for the formation of flavonoid derivatives with multiple organic acid substitutions – here malic acid - as we have seen that in the HSCCC-ESI-MS coupling experiment.

Furthermore, we also detected acetate, malonate and di-oxalic acid substitutions in the flavonoid structures by characteristic ESI-MS/MS fragment ions and neutral loss calculations. Interestingly, the di-oxalyl- and malonyl-derivatives of other polyphenols such as anthocyanins are known to be quite unstable and are rather difficult to isolate. We postulated that the malic acid ester substitution preferably occurs at position C-6 of the hexose (glucose) unit, due to increased accessability and less steric hindrance of the respective hydroxyl-group. Finally, more experiments will be helpful to evaluate the possibilities to use these thermally generated ‘artefacts’ as marker substances to distinguish direct bottled juices from concentrate by comparing the profile of genuine flavonoids and novel acid substituted flavonoid glycosides, and the influence of the HTST process (high-temperature-short-time treatment) to the formation of malyl-flavonoid esters in juices and concentrates.

In summary, on-line coupling of preparative HSCCC with ESI-MS/MS had shown the versatile potentials for performing large scale isolation and meanwhile providing necessary informations about the structures of the eluted compounds to perform a *target-guided* and more focussed isolation of the desirable and potentially unknown natural products from any crude plant extracts. Future work could be focused on the investigation of unknown compounds in high acidic fruit juices and the related concentrate products. This would be an interesting challenge to screen for further thermally changed polyphenols – such as esterified flavonoids and most likely anthocyanin-glycoside derivatives in the deeply red-colored juice concentrates. The kinetics and stabilities of the acyl-flavonoids are completely unknown and their fate over storage time could be investigated after reconstitution of juice concentrates to the final juice products.

References

1. Editorial Board of Pharmacopoeia of the P.R. China. *Pharmacopoeia of the Peoples Republic of China*; Peoples Medical Publishing House: Beijing, 1977.
2. Teng, B. S.; Lu, Y. H.; Wang, Z. T.; Tao, X. Y.; Wei, D. Z. *In vitro* anti-tumor activity of isorhamnetin isolated from *Hippophae rhamnoides* L. against BEL-7402 cells. *Pharmacol. Res.* **2006**, *54*, 186–194.
3. Zeb, A. Important therapeutic uses of sea buckthorn (*Hippophae*): A review. *J. Biol. Sci.* **2004a**, *4*, 687–693.
4. Zeb, A. Chemical and nutritional constituents of sea buckthorn juice. *Pakistan J. Nutr.* **2004b**, *3*, 99–106.
5. Guliyev, V. B.; Gul, M.; Yildirim, A. 2004. *Hippophae rhamnoides* L.: Chromatographic methods to determine chemical composition, use in traditional medicine, and pharmacological effects. *J. Chromatogr., B* **2004**, *812*, 291–307.
6. Ito, Y.; Conway, W. D., Eds. *High-Speed Countercurrent Chromatography*, Chemical Analysis 132; Wiley: New York, 1996.
7. Ito, Y. Golden rules and pitfalls in selecting optimum conditions for high-speed counter-current chromatography. *J. Chromatogr., A* **2005**, *1065*, 145–168.
8. Oka, H.; Ikai, Y.; Kawamura, N.; Hayakawa, J.; Harada, K. I.; Murata, H.; Suzuki, M.; Ito, Y. Direct interfacing of high-speed countercurrent chromatography to frit electron ionization, chemical ionization, and fast atom bombardement mass spectrometry. *Anal. Chem.* **1991**, *63*, 2861–2865.
9. Kong, Z.; Rinehart, K. L.; Milberg, R. M.; Conway, W. D. Application of high-speed countercurrent chromatography/electrospray ionization mass spectrometry (HSCCC/ESIMS) in natural products chemistry. *J. Liq. Chromatogr. Relat. Technol.* **1998**, *21*, 65–82.
10. Lee, Y. W.; Yoyksner, R. D.; Fang, Q. C.; Cook, C. E.; Ito, Y. Application of countercurrent chromatography/thermospray mass spectrometry for the analysis of natural products. *J. Liq. Chromatogr.* **1988**, *11*, 153–171.
11. Janaway, L.; Hawes, D.; Ignatova, S.; Wood, P.; Sutherland, I.A. A new small coil-volume CCC instrument for direct interfacing with MS. *J. Liq. Chromatogr. Relat. Technol.* **2003**, *26*, 1345–1354.
12. Gutzeit, D.; Winterhalter, P.; Jerz, G. Application of preparative high-speed counter-current chromatography/electrospray ionization mass spectrometry

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for a fast screening and fractionation of polyphenols. *J. Chromatogr., A* **2007**, *1172*, 40–46.

13. Rösch, D.; Bergmann, M.; Knorr, D.; Kroh, L. W. Structure-antioxidant efficiency relationships of phenolic compounds and their contribution to the antioxidant activity of sea buckthorn juice. *J. Agric. Food Chem.* **2003**, *51*, 4233–4239.
14. Tiitinen, K. M.; Hakala, M. A.; Kallio, H. P. Quality components of sea buckthorn (*Hippophaë rhamnoides*) varities. *J. Agric. Food Chem.* **2005**, *53*, 1692–1699.
15. Gutzeit, D.; Wray, V.; Winterhalter, P.; Jerz, G. Preparative isolation and purification of flavonoids and protocatechuic acid from sea buckthorn juice concentrate (*Hippophaë rhamnoides* L. ssp. *rhamnoides*) by high-speed counter-current chromatography. *Chromatographia* **2007**, *65*, 1–7.

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Chapter 17

Preparative Isolation of Bioactive Constituents from Berries

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For studies concerning the bioavailability and bioactivity of berry constituents purified substances in the gram-scale are required. This chapter will present different strategies for the purification and preparation of bioactives from berries which include different types of the all-liquid chromatographic technique of countercurrent chromatography as well as semisynthetic strategies. Berries investigated are *inter alia* blackberries, black chokeberries, and grapes. The target compounds include anthocyanins, stilbenes, and proanthocyanins.

Initial screenings for health promoting activities of berries can often be carried out with crude natural extracts. However, it is very likely that the investigation comes to a point where the biologically active compound has to be isolated from a complex mixture of other plant constituents. In many cases this isolation step is time consuming and often it is simply impossible to obtain the target compound in sufficient amounts for subsequent studies concerning bioactivity, bioavailability, and metabolic fate. During the last years, countercurrent chromatography (CCC) was recognized as a versatile tool in natural product analysis. CCC is one of the few liquid chromatographic techniques that can be predictably scaled up from analytical to process scale. In combination with the 100 % recovery of the sample and the gentle separation conditions, this technique is especially suited to the analysis of phenolic berry constituents, such as anthocyanins and other polar flavonoids. Although CCC is a versatile technique - even suitable for the enrichment of trace constituents – applications are known where CCC

In Flavor and Health Benefits of Small Fruits; Qian, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

purifications/fractionations have to be combined with a semi-synthetic approach. As an example the preparation of dimeric procyanidins from a polyphenolic fraction of aronia will be presented.

Principle of Countercurrent Chromatography (CCC)

The modern era of CCC began with the development by Y. Ito of the coil planet centrifuge (*1*, *2*), a technique which was marketed as multilayer coil countercurrent chromatography (MLCCC) or high speed countercurrent chromatography (HSCCC). In the following, the working principles of these techniques can only be briefly outlined, for a more detailed description the reader is referred to various monographs on countercurrent chromatography (*3–5*). In MLCCC as well as in HSCCC the separating column consists of a PTFE tubing that is wrapped around a holder in several layers (multilayer coil). The radius **r** of the coiled column depends on the number of layers. In essence, the coil undergoes a synchronous planetary motion while the column holder revolves around the central axis of the apparatus. The revolution radius is **R**, and the ratio of **r/R** is defined as *ß-value*, which may vary from 0.25 to 0.8. The apparatus is designed with an anti-twist mechanism that ensures continuous solvent flow without requiring a rotating seal. During rotation, an Archimedean screw force is created causing the migration of the stationary phase towards one end of the column. The mobile phase is now introduced the opposite direction. During operation the interfacial friction force and the Archimedean screw force are counteracting and create a *hydrodynamic equilibrium* depending on the rotation speed, flow rate, ß-value and viscosity of the solvent. Ideally, the mobile phase can be pumped through the system with the stationary phase being almost completely retained in the system.

Partitioning of the solutes through mixing of the two immiscible phases is automatically achieved. When the ß-value exceeds 0.5 the trajectory forms a loop in which the force field is much lower compared with the opposite part of the loop. This difference leads to the following behavior of a two phase system in the coil: when the force field is strong the phases are separated (settling step), when the force field is weak (in the loop) mixing of the two phases occurs (mixing step). Injection of a crude sample can result in up to 50 000 liquid-liquid partition steps per hour as it successively passes through alternate settling and mixing zones of the coiled column, thus enabling an efficient partition chromatography.

CCC can either be used as fractionation technique enabling subsequent testing of the fractions for certain biological activities. Alternatively, CCC can be used as an isolation tool. Especially in cases where major plant constituents need to be isolated, preparative amounts of pure substances are often accessible in a single CCC run. Separations by MLCCC or HSCCC are usually done on a 1-3 gram scale. For the separation of higher amounts (up to 100 g and higher), the so-called Low Speed Rotary Countercurrent Chromatography (LSRCCC) has been developed. In LSRCCC a cylindrical column rotates slowly around a single axis. Du and coworkers (*6*, *7*) have shown that the use of special convoluted tubing

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enabled sufficient retention of stationary phase at a rotational speed of only 50-100 rpm. LSRCCC is readily scaled up (kg scale) by using longer columns and/or by increasing the inner diameter of the convoluted tubing (*8*, *9*).

Applications of Countercurrent Chromatography

Separation of Anthocyanin Mixtures Using HSCCC

Berry anthocyanins exhibit a range of biological activities and their intake may help to improve or at least maintain human health. One of the best known attributes of anthocyanins is the antioxidant activity, especially of the cyanidin derivatives. Other more specific bioactivities are still under active investigation and for intervention studies carried out with individual anthocyanins large amounts of purified testing substances are required. There are multiple applications of CCC in the field of anthocyanin analysis with clear advantages over conventional techniques. CCC provides a rapid fractionation tool for crude mixtures which *inter alia* leads to an enrichment of minor constituents in the separated CCC fractions. Rechromatography with CCC or preparative HPLC then gives ready access to pure pigments. A typical biphasic solvent mixture for anthocyanin separation is based on the following four solvents: MTBE/*n*-butanol/acetonitrile/water (in varying relative proportions) acidified with a small amount of TFA. Numerous applications for the separation of the anthocyanin mixtures from e.g. elderberry, sour cherry, billberry are known (*10*–*12*). In combination with LC-NMR, HSCCC has been used to identify the so far unknown anthocyanin composition of tayberry (*Rubus loganobaccus*, cross of blackberry x raspberry). The anthocyanin extract was fractionated with the biphasic solvent system MTBE/n-butanol/acetonitrile/water (1:3:1:5, v/v/v/v) and the obtained HSCCC fractions were directly analyzed by LC-NMR. In this way, five cyanidin derivatives (i.e. cyanidin 3-glucoside, cyanidin 3-rutinoside, cyanidin 3-sophoroside, cyanidin 3-glucosylrutinoside, cyanidin 3-sambubioside) could be identified without any additional workup of the sample (*13*).

Low-Speed Rotary Countercurrent Chromatography (LSRCCC) Separation of Anthocyanins from Blackberries

LSRCCC is the method of choice for preparative separations of natural products in the 10-500 g scale. Applied to blackberry anthocyanins (cf. Figure 1), LSRCCC allowed the purification of 30 grams of cyanidin 3-glucoside, which was *inter alia* used for a colon cancer study (*14*). The intestinal adenoma formation in the Apc^{Min} mouse (a genetic model for human familial adenomatous polyposis) was reduced dose-dependently by the ingested cyanidin 3-glucoside. It was found that at the highest dose (0.3 % of cyanidin 3-glucoside in the diet for twelve weeks) adenoma numbers were decreased by 45 %.

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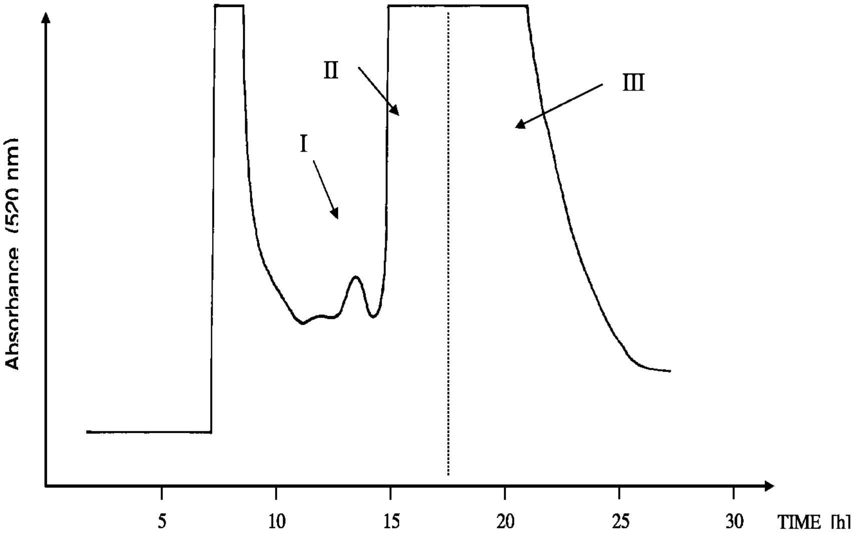

Figure 1. Fractionation of a blackberry extract (30 g) using LSRCCC. Solvent system MTBE/n-butanol/acetonitrile/water (2:2:1:5, v/v/v/v, acidified with 0.1% TFA). Fraction I: Cyanidin-3-rutinoside, Fraction II: pure Cyanidin-3-glucoside (8.6 g), Fraction III: Cyanidin-3-glucoside plus two impurities.

Stilbenes in *Vitis vinifera*

Stilbenes are nonflavonoid phenolics that are mainly present in grapes and derived products, such as grape juice and wine (*15*). As phytoalexins they are biosynthesized by grapevine as a defence response to biotic or abiotic stress. Due to their antioxidative, anticarcinogenic, and potentially life prolonging activities, stilbenes are considered to play an important role in the human diet. More than 30 stilbenes and stilbene glycosides occur naturally in various plant species. Their structural nucleus is based on a 14-carbon skeleton composed of two phenyl rings joined by an ethylene bridge. Figure 2 shows the chemical structures of several stilbenes. One of the most well known and widely distributed stilbenes is reveratrol, a 3,4′,5-tri-hydroxystilbene. In nature, resveratrol exists in two isomeric forms (*cis* and *trans*-configured) either ß-glycosylated or as aglycone. Trans-resveratrol occurs in various plant families. Especially in fruits like blueberry, cranberry, cowberry, mulberry and jackfruit resveratrol and its glycoside are present as minor components. The highest concentrations are detected in grapes and derived products. Resveratrol is not present in grape berry flesh, but only in the skins and seeds. A multitude of studies have examined the potential health effects of the consumption of resveratrol by humans. Resveratrol is supposed to prevent and reduce a wide range of diseases such as cancer, cardiovascular diseases, and ischemic damage. It may inhibit the carcinogenesis at multiple stages, such as tumor initiation, promotion, and progression (*16–18*).

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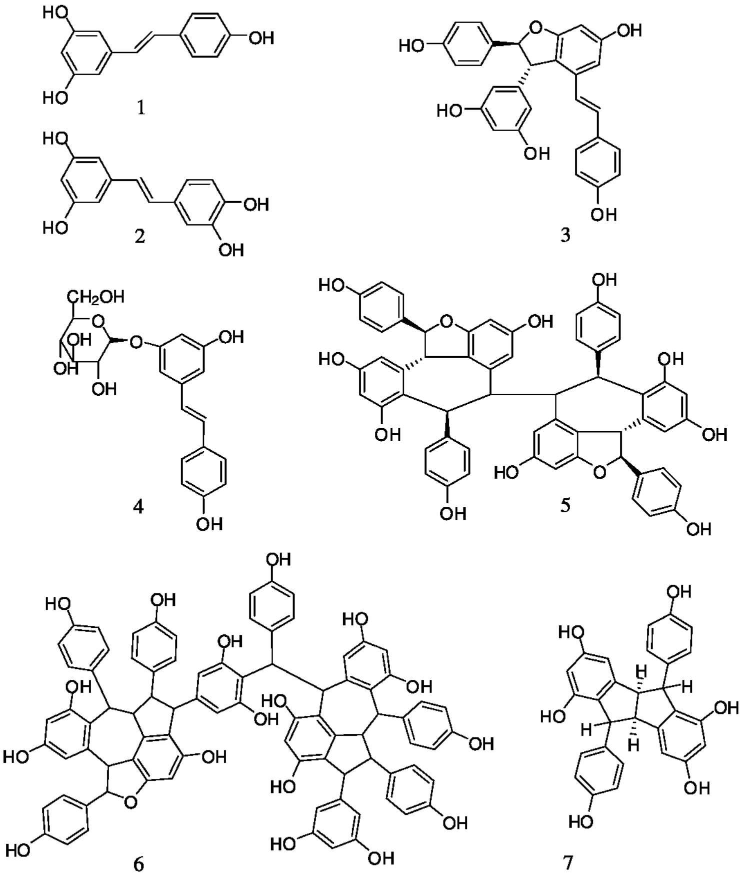

Figure 2. Chemical structures of stilbenes: trans-resveratrol (1), trans-piceatannol (2), trans-ε-viniferin (3), trans-piceid (4), (+)-hopeaphenol (5), vaticanol D (6), pallidol (7)

The amount of stilbenes in grapes and wine varies widely depending on many factors such as geographic region, agronomic factors, climatic factors, plant stress conditions and oenological practices. Because of the contact of grapeskin and seeds during the fermentation process, the concentration of resveratrol in red wine (0.2-13 mg/L) is higher than in white wine (0.1-0.8 mg/L). The level of resveratrol found in rosé wine ranges between the levels of red and white wine (*19*). For piceid, the resveratrol-3-*O*-glucoside, the concentrations are reported to be in a range of 0.3-9 mg/L in red and 0.1-2.2 mg/L in white wine (*15*, *20*).

Stilbenes also occur in oligomeric forms, so-called viniferins. Their biosynthesis includes an oxidative polymerization of the monomer resveratrol as result of the activities of peroxidases (*21*). So far, resveratrol oligomers ranging from dimer to octamer are known. Some of these stilbene oligomers exhibit antibacterial, anti-HIV, anti-inflammatory, antioxidant, and antitumor activities (*22*, *23*). In a survey of commercial wines from the South of France, levels of pallidol and ε-viniferin have been reported. Viniferin was found to be present in red and botrytized sweet white wines in levels between 0.1 and 1.63 mg/L. Pallidol was found in wines made by maceration with stems, in levels between 0.38 and 2.22 mg/L (*24*).

Separation of Oligomeric Stilbenes from *Vitis vinifera* Using MLCCC/HSCCC

Due to the low concentration of oligomeric stilbenes in grapes and wines isolation of sufficient amounts for subsequent bioactivity studies is difficult to achieve. After a work-up of 100 L of a commercial Riesling wine, fractionation with MLCCC and purification with preparative HPLC, the structure of novel stilbenes could be elucidated. The quantity of the isolated substances however was only in the mg-range (*25*, *26*). As more promising source for the isolation of oligomeric stilbenes, grapevine shoots — a by-product of viticulture — can be regarded. In recent studies, a commercial grapevine extract was fractionated by High-Speed Countercurrent Chromatography (HSCCC). The major stilbenes of the extract were *trans*-resveratrol, *trans*-ε-viniferin, trans-piceatannol and the tetramer r-viniferin. In Figure 3 the HPLC-PDA chromatogram of the raw grapevine extract at 306 nm and the structure of r-viniferin are displayed.

After HSCCC separation of the grapevine extract (sample quantity 2.8 g, separation time 9.5 h) 14 fractions were obtained. Based on HPLC-ESI-MS^n analyses, *trans*-resveratrol and *trans*-ε-viniferin were identified as major constituents. By using preparative HPLC it was possible to obtain preparative amounts of *trans*-resveratrol (61.5 mg) and *trans*-ε-viniferin (80.7 mg) in a purity > 95 %.

Possibilities for Preparative Isolations of Dimeric Procyanidins

Procyanidins are widely found as secondary metabolites in plants. They provide the bitter and astringent taste and are important for color as well as for flavor of beverages and foods (*27*). The procyanidins, one subclass of proanthocyanidins, are mixtures of dimers, oligomers, and polymers consisting of the flavan-3-ols (+)-catechin and/or (-)-epicatechin. Mostly, the linkage is between C_4 of the upper unit and C_8 of the lower unit (procyanidins B1-B4), rarely between C_4 of the upper unit and C_6 of the lower unit (procyanidins B5-B8). Figure 4 shows the chemical structures of dimeric procyanidins. Procyanidins are present in several beverages and foods for example tea, wine, cocoa, grape, and fruit juices (*28*). The lower molecular weight procyanidins are usually present in plant tissue in relatively low concentrations compared to that of higher oligomers and polymers (*29*). The molecular weight of procyanidins is expressed as degree of polymerization (DP). According to DP, procyanidins are classified in three groups. Besides the monomers (DP = 1), oligomeric (DP = 2-10) and polymeric (DP >10) procyandins are distinguished.

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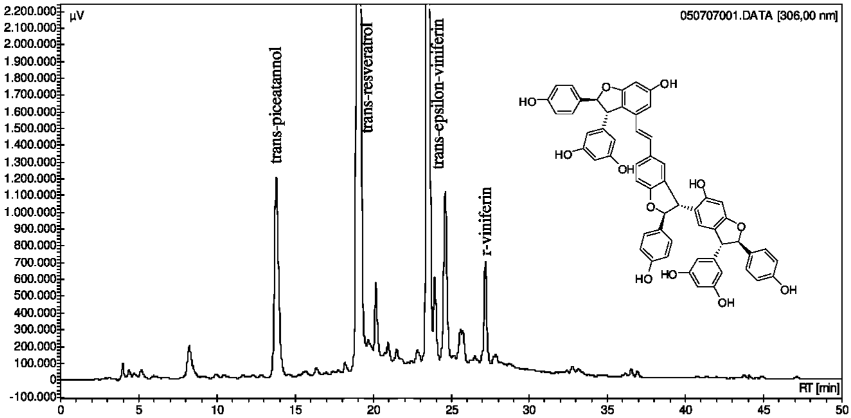
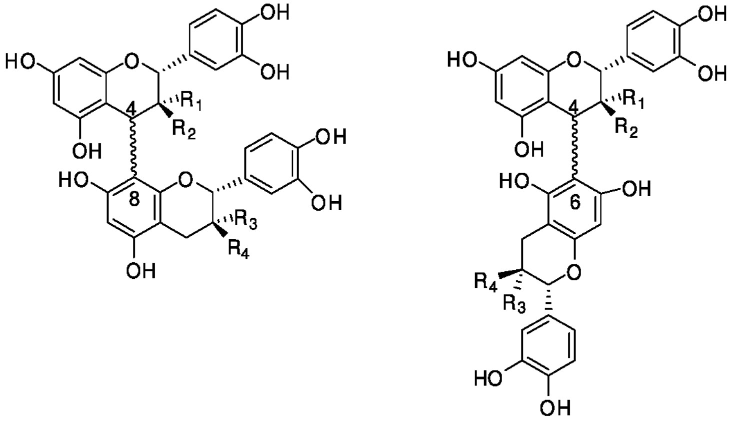


Figure 3. HPLC-PDA chromatogram of a grapevine extract at 306 nm and the structure of r-viniferin


OH
HO
O
OH
4
R1
R2
OH
OH
HO
O
8
R3
R4
OH
OH
OH
OH
HO
O
4
R1
R2
OH
HO
6
OH
R4
R3
O
HO
OH

	R_1	R_2	R_3	R_4
B1	OH	H	H	OH
B2	OH	H	OH	H
B3	H	OH	H	OH
B4	H	OH	OH	H

	R_1	R_2	R_3	R_4
B5	OH	H	OH	H
B6	H	OH	H	OH
B7	OH	H	H	OH
B8	H	OH	OH	H

Figure 4. Chemical structures of dimeric procyanidins

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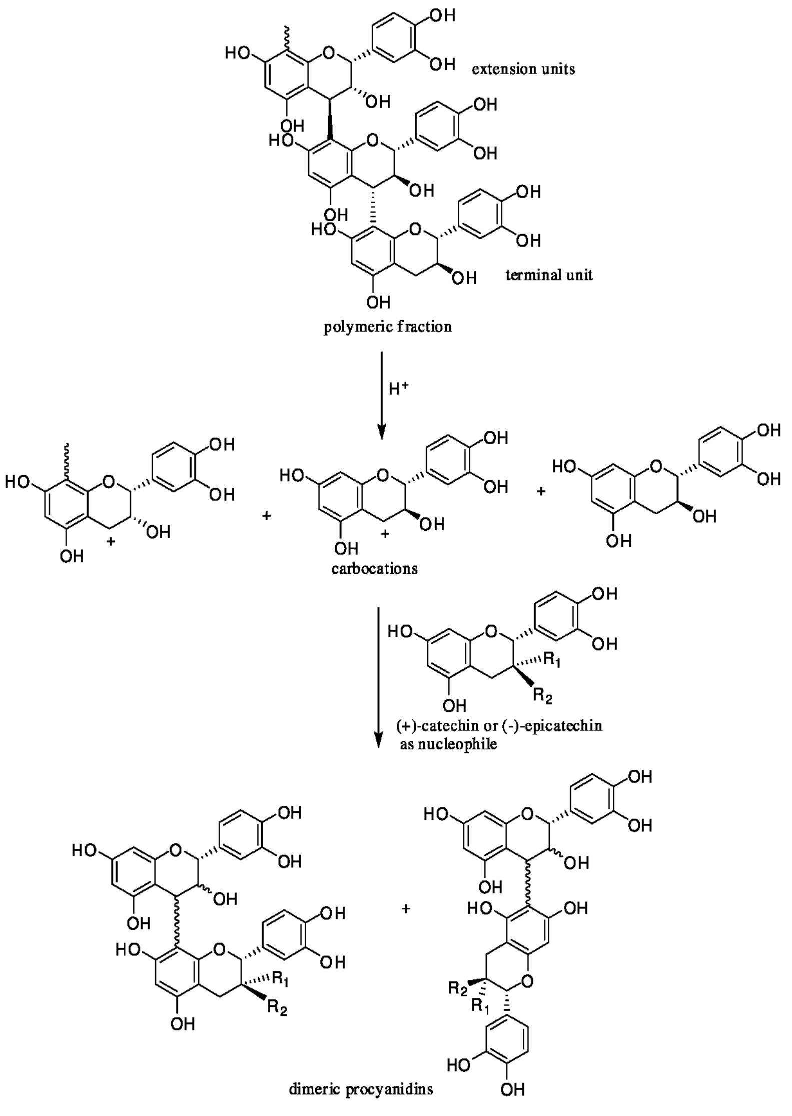
extension units
terminal unit
polymeric fraction
H+
carbocations
(+)-catechin or (-)-epicatechin
as nucleophile
dimeric procyanidins

Figure 5. Mechanism of semisynthesis

Grape Seed Extract (*Vitis vinifera*) and Cocoa Beans (*Theobroma cacao* L.) Present Good Sources for the Isolation of Dimeric Procyanidins

Grape seed extracts are good sources for the isolation of dimeric procyanidins with the concentrations of B1, B2, B3, and B4 being in the range of 1.2 to 77.2 mg/g (*30*). High speed countercurrent chromatography (HSCCC) has been successfully used for the isolation of grape seed procyanidins on a semi-preparative scale. The isolation of dimeric to tetrameric procyanidins was

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achieved after removing the polymeric procyanidins by solvent precipitation which is essential for a successful HSCCC separation. The filtrate was enriched with low molecular oligomeric procyanidins especially with dimers and the precipitate was composed of higher oligomeric procyanidins. By using various biphasic solvent systems, dimeric procyanidins B1, B2, B3, B4, B5, and B7, as well as trimeric C1 and tetrameric A2 were obtained in high purities (*31*).

In the case of cocoa beans (proanthocyanidin content approx. 58%) only procyanidins with (-)-epicatechin as the main extension subunit, such as dimeric B1, B2, B4, B5, and trimeric C1 were detected (*32*). As before for a successful HSCCC separation, elimination of polymeric compounds by solvent precipitation is required. In this way it is possible to obtain the dimeric procyanidins B2 and B5 as well as the trimer C1 in high purity (*33*).

Black Chokeberry (*Aronia melanocarpa*)

Aronia berry, also called black chokeberry, is a member of the *Rosaceae* family. The plant originates from the eastern parts of North America and East Canada and was introduced in Europe at the beginning of the 20th century. *Aronia melanocarpa* is a 2–3 m high shrub, which produces in May to June white flowers. Harvest of chokeberries is between August and September (*34*). The berries are known to contain flavonoids such as anthocyanins and proanthocyanidins, phenolic acids, stilbens and lignans. Polymeric procyanidins are the major class of polyphenolic compounds, whereas non-acylated cyanidin-based anthocyanins are the second phenolic compound group in chokeberry. Aronia berries are also rich in phenolic acids like neochlorogenic acid and chlorogenic acid and different quercetin 3-glycosides derivates (*35*, *36*).

Because of the anthocyanins and proanthocyanidins, the berries have a high antioxidant activity, which has a positive effect on the human health (*37*, *38*). Due to the reduction of the antioxidative stress, many diseases like atherosclerosis are affected in this way. Chokeberries have also antimutagenic, hepatoprotective, cardioprotective, antidiabetic and protective effects against colon cancer (*34*).

Procyanidins in Chokeberries

Aronia contains exclusively B-type PCs with (-)-epicatechin as the main subunit monomer (*35*). Our results have shown that they contain traces of dimeric procyanidins like B1, B2, B7, and B5, also monomeric (-)-epicatechin and trimeric procyanidin C1. High amounts of polymeric procyanidins in chokeberries were determined to be 5182 mg/100 g DW (dry weight) (*35*). With regard to the composition of the polymers, phloroglucinolyses revealed that they almost exclusively consist of (-)-epicatechin units, the portion of (+)-catechin units is about 1.5%. However, galloylated subunits were not detected until now (*34*).

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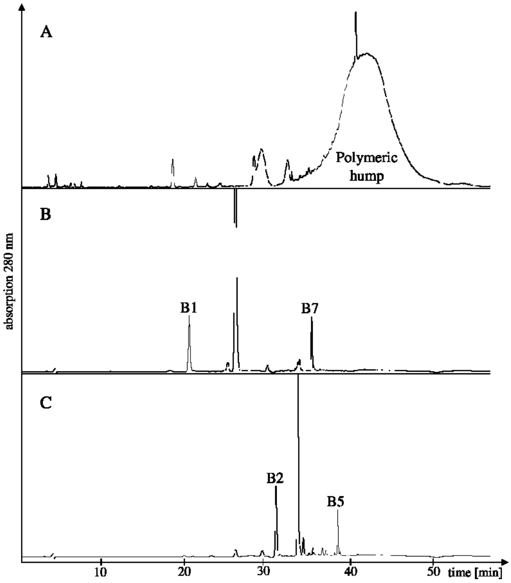

Figure 6. (A): HPLC-PDA analysis of the aronia seed precipitate before semisynthesis; (B, C): HPLC-PDA analysis of the aronia seed precipitate after semisynthesis with (+)-catechin (B) or (-)-epicatechin (C) as nucleophile under conditions above (cf. text)

Semisynthesis of Dimeric Procyanidins

In contrast to grape seed extracts and cocoa beans, it is not possible to isolate dimeric procyanidins directly from chokeberries by HSCCC. Only recently, a semisynthetic approach has been developed for the preparative formation of dimeric procyanidins from polymeric procyanidins. Under acid conditions the interflavanoid linkage of polymeric procyanidins is cleaved and releases epicatechin as carbocation and terminal unit. This carbocation can react with the terminal unit or with a nucleophil, in this case (+)-catechin or (-)-epicatechin (cf. Figure 5). In this way, the polymeric procyanidins are degraded while dimeric procyanidins are formed (*39*).

Moreover, aronia seed precipitate can be used as starting material for the semisynthesis of procyanidins B1, B2, B5, and B7. This polymeric fraction can be obtained by precipitation of the acetone extract from aronia seeds with ethanol and *n*-hexane. The aronia seed precipitate consists of polymers that have (-)-epicatechin in the upper and terminal unit. For this reason, during the semisynthesis only dimeric procyanidins that contain epicatechin in the upper unit, like B1, B2, B5, and B7 are formed. With (+)-catechin as nucleophile B1 and B7 are formed while the addition of (-)-epicatechin produces B2 and B5 (cf. Figure 6).

In addition to procyanidin formation, by-products like chalcane flavan-3-ol dimers, the so-called gambiriins, are formed. The influence of reaction time, temperature, and ratio of reactants like nucleophile and aronia seed precipitate was examined to find optimal reaction conditions. Our analyses revealed the following optimized conditions for semisynthesis: temperature of 40 °C, reaction time of 20 min and reactants in a ratio of 2:1. Thereby, it was possible to form pure dimeric B1, B2, B5 and B7 on a preparative scale with HSCCC.

These examples show that multiple strategies will lead to the isolation of pure berry constituents. In many cases application of CCC — alone or in combination with synthetic approaches — will give access to the target compounds in amounts that will allow subsequent testing of biological activities. With the availability of novel CCC instrumentation, such as LSRCCC, isolation of pure compounds even on the 100 gram scale becomes possible. Intervention studies with these well defined constituents will open new avenues for bioactivity studies and finally shed more light onto the health benefits of phenolic berry constituents.

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References

1. Ito, Y. Efficient preparative countercurrent chromatography with a coil planet centrifuge. *J. Chromatogr.* **1981**, *214*, 122–125.
2. Ito, Y. High-speed countercurrent chromatography. *CRC Crit. Rev. Anal. Chem.* **1986**, *17*, 65–143.
3. *Modern Countercurrent Chromatography*; Conway, W. D.; Petroski, R. J., Eds.; ACS Symposium Series 593; American Chemical Society: Washington, DC, 1995.
4. *Countercurrent Chromatography: Theory and Practice*; Mandava, N. B.; Ito, Y., Eds.; Marcel Dekker: New York, 1988.
5. Conway, W. D. *Countercurrent Chromatography: Apparatus, Theory, and Applications*; VCH Publishers, Inc.: New York, 1990.
6. Du, Q.; Wu, P.; Ito, Y. Low-speed rotary countercurrent chromatography using a convoluted multilayer helical tube for industrial separation. *Anal. Chem.* **2000**, *72*, 3363–3365.
7. Du, Q.; Winterhalter, P.; Ito, Y. Large convoluted tubing for scale-up of slow rotary countercurrent chromatograph. *J. Liq. Chromatogr. Relat. Technol.* **2003**, *26*, 1991–2001.
8. Du, Q.; Jerz, G.; He, Y.; Li, L.; Xu, X.; Zhang, Q.; Zheng, Q.; Winterhalter, P.; Ito, Y. Semi-industrial isolation of salicin and amygdalin from plant extracts using slow rotary counter-current chromatography. *J. Chromatogr., A* **2005**, *1074*, 43–46.
9. Köhler, N.; Chou, E.; Ito, Y.; Winterhalter, P. Development of a new preparative spiral-coil low-speed rotary countercurrent chromatographic (spiral-coil LSRCCC) method. *J. Liq. Chromatogr. Relat. Technol.* **2004**, *27*, 2547–2560.
10. Degenhardt, A.; Winterhalter, P. Isolation of natural pigments by high speed CCC. *J. Liq. Chromatogr. Relat. Technol.* **2001**, *24*, 1745–1764.

11. Schwarz, M.; Hillebrand, S.; Habben, S.; Degenhardt, A.; Winterhalter, P. Application of high-speed countercurrent chromatography to the large-scale isolation of anthocyanins. *Biochem. Eng. J.* **2003**, *14*, 179–189.
12. Winterhalter, P. Application of countercurrent chromatography (CCC) to the analysis of natural pigments. *Trends Food Sci. Technol.* **2007**, *18*, 507–513.
13. Hillebrand, S. Ph.D. Thesis, TU Braunschweig, Cuvillier: Göttingen, Germany, 2004, ISBN 3-86537-289-9.
14. Cooke, D.; Schwarz, M.; Boocock, D.; Winterhalter, P.; Steward, W. P.; Gescher, A. J.; Marczylo, T. H. Effect of cyanidin-3-glucoside and an anthocyanin mixture from bilberry on adenoma development in the *Apc*^{Min} mouse model of intestinal carcinogenesis: Relationship with tissue anthocyanin levels. *Int. J. Cancer* **2006**, *119*, 2213–2220.
15. Lamuela-Raventós, R. M.; Romero-Pérez, A. I.; Waterhouse, A. L.; de la Torre-Boronat, M. C. Direct HPLC analysis of *cis*- and *trans*-resveratrol and piceid isomers in Spanish red *Vitis vinifera* wines. *J. Agric. Food Chem.* **1995**, *43*, 281–283.
16. Jang, M.; Cai, L.; Udeani, G. O.; Slowing, K. V.; Thomas, C. F.; Beecher, C. W.; Fong, H. H.; Farnsworth, N. R.; Kinghorn, A. D.; Mehta, R. G.; Moon, R. C.; Pezzuto, J. M. Cancer chemopreventive activity of resveratrol: A natural product derived from grapes. *Am. Assoc. Adv. Sci.* **1997**, *275*, 218–220.
17. Aggarwal, B. B.; Bhardwaj, A.; Aggarwal, R. S.; Seeram, N. P.; Shishodia, S.; Takada, Y. Role of resveratrol in prevention and therapy of cancer: Preclinical and clinical studies. *Anticancer Res.* **2004**, *24*, 2783–2840.
18. Guerrero, R. F.; Garcia-Parrilla, M. C.; Puertas, B.; Cantos-Villar, E. Wine, resveratrol, and health: A review. *Nat. Prod. Commun.* **2009**, *4*, 635–658.
19. Stecher, G.; Huck, C. W.; Popp, M.; Bonn, G. K. Determination of flavonoids and stilbenes in red wine and related biological products by HPLC and HPLC-ESI-MS-MS. *Fresenius J. Anal. Chem.* **2001**, *371*, 73–80.
20. Goldberg, D. M.; Yan, J.; Ng, E.; Diamandis, E. P.; Karumanchiri, A.; Soleas, G.; Waterhouse, A. L. A global survey of *trans*-resveratrol concentrations in commercial wines. *Am. J. Enol. Vitic.* **1995**, *46*, 159–165.
21. Takaya, Y.; Terashima, K.; Ito, J.; He, Y. H.; Tateoka, M.; Yamaguchi, N.; Niwa, M. Biomimic transformation of resveratrol. *Tetrahedron* **2005**, *61*, 10285–10290.
22. Cichewicz, R. H.; Kouzi, S. A. Resveratrol oligomers: Structure, chemistry, and biological activity. In *Studies in Natural Products Chemistry, Bioactive Natural Products (Part G)*; 2002; Vol. 26, pp 507−579.
23. Ito, T.; Akao, Y.; Yi, H.; Ohguchi, K.; Matsumoto, K.; Tanaka, T.; Iinuma, M.; Nozawa, Y. Antitumor effect of resveratrol oligomers against human cancer cell lines and the molecular mechanism of apoptosis induced by vaticanol C. *Carcinogenesis* **2003**, *24*, 1489–1497.
24. Landrault, N.; Larronde, F.; Delaunay, J.-C.; Castagnino, C.; Vercauteren, J.; Mérillon, J.-M.; Gasc, F.; Cros, G.; Teissédre, P.-L. Levels of stilbene oligomers and astilbin in French varietal wines and in grapes during noble rot development. *J. Agric. Food Chem.* **2002**, *50*, 2046–2052.

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25. Baderschneider, B.; Winterhalter, P. Isolation and characterization of novel stilbene derivatives from Riesling wine. *J. Agric. Food Chem.* **2000**, *48*, 2681–2686.
26. Rentzsch, M.; Wilkens, A.; Winterhalter,P. Non-flavonoid phenolic compounds. In *Wine Chemistry and Biochemistry*; Polo, C., Moreno-Arribas, M. V., Eds.; Springer: New York, 2008, pp 509−527.
27. Shoji, T.; Mutsuga, M.; Nakamura, T.; Kanda, T.; Akiyama, H.; Goda, Y. Isolation and structural elucidation of some procyanidins from apple by low-temperature nuclear magnetic resonance. *J. Agric. Food Chem.* **2003**, *51*, 3806–3813.
28. Dixon, R. A.; Xie, D.-Y.; Sharma, S. Proanthocyanidins—A final frontier in flavonoid research?. *New Phytol.* **2005**, *165*, 9–28.
29. Czochanska, Z.; Foo, L. Y.; Newman, R. H.; Porter, L. J. Polymeric proanthocyanidins. Stereochemistry, structural units, and molecular weight. *J. Chem. Soc., Perkin Trans. 1* **1980**, 2278–2286.
30. Monagas, M.; Hernandez-Ledesma, B.; Garrido, I.; Martin-Alvarez, P. J.; Gomez-Cordoves, C.; Bartolome, B. Quality assessment of commercial dietary antioxidant products from *Vitis vinifera* L. grape seeds. *Nutrition and Cancer* **2005**, *53*, 244–254.
31. Köhler, N.; Wray, V.; Winterhalter, P. Preparative isolation of procyanidins from grape seed extracts by high-speed counter-current chromatography. *J. Chromatogr., A* **2008**, *1177*, 114–125.
32. Wollgast, J.; Anklam, E. Review on polyphenols in *Theobroma cacao*: Changes in composition during the manufacture of chocolate and methodology for identification and quantification. *Food Res. Int.* **2000**, *33*, 423–447.
33. Köhler, N. Ph.D. Thesis, TU Braunschweig, Braunschweig, Germany, 2006.
34. Kulling, S. E.; Rawel, H. Chokeberry (*Aronia melanocarpa*): A review on the characteristic components and potential health effects. *Planta Med.* **2008**, *74*, 1625–1634.
35. Oszmianski, J.; Wojdylo, A. *Aronia melanocarpa* phenolics and their antioxidant activity. *Eur. Food Res. Technol.* **2005**, *221*, 809–813.
36. Jakobek, L.; Seruga, M.; Mevidovic-Kosanovic, M.; Novak, I. Anthocyanin content and antioxidant activity of various red fruit juices. *Dtsch. Lebensm.-Rundsch.* **2007**, *103*, 58–64.
37. Aron, P. M.; Kennedy, J. A. Flavan-3-ols: Nature, occurrence and biological activity. *Mol. Nutr. Food Res.* **2008**, *52*, 70–104.
38. Zafra-Stone, S; Yasmin, T.; Bagchi, M.; Chatterjee, A.; Vinson, J. A.; Bagchi, D. Berry anthocyanins as novel antioxidants in human health and disease prevention. *Mol. Nutr. Food Res.* **2007**, *51*, 675–683.
39. Köhler, N.; Wray, V.; Winterhalter, P. New approach for the synthesis and isolation of dimeric procyanidins. *J. Agric. Food Chem.* **2008**, *56*, 5374–5385.

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Chapter 18

Edible Coatings for Enhancing Quality and Health Benefits of Berry Fruits

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Edible coatings may be used on the surface of fresh berry fruits to modify the internal atmosphere, decrease the transpiration loss, and delay the ripening during postharvest storage and handling. Meanwhile, certain coating materials, such as chitosan has strong antifungal ability against *Botrytis cinerea* and *Rhizopus* sp., the two main fungi causing the decay of berry fruits. Therefore, edible coating technology, when designed and applied correctly, is an effective means to control decay and extend shelf-life of some berry fruits. In addition, edible coatings may provide an excellent vehicle to further enhance health benefit of berry fruits where the lack of some important nutraceuticals, such as vitamin E and calcium may be compensated by incorporating them into the coatings. This chapter briefly discusses the concepts and means of applying edible coatings on the surface of berry fruits and gives a review of the research conducted in the author's laboratory in the development of edible coatings and their demonstrations on a broad range of berry fruits, including strawberries, raspberries, hardy kiwifruits, and blueberries.

Edible Coatings and Their Application on Berry Fruits

Edible coatings are the thin layers of edible materials formed on the surface of food by casting, dipping, spraying, or brushing. They offer a selective barrier against the transmission of gases, water vapor, and solutes while also providing

In Flavor and Health Benefits of Small Fruits; Qian, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

mechanical protection (Figure 1). By regulating the transfer of moisture, oxygen, carbon dioxide, aroma and flavor compounds in a food system, edible coatings have demonstrated the capability of improving food quality and prolonging shelf-life of fresh fruits and vegetables, including berry fruits. Edible coatings may also be used to advantage on processed fruits and vegetables for improving structural integrity of frozen fruits and vegetables and preventing moisture absorption and oxidation of dried fruits or vegetables (*1*). In addition, edible coatings can provide active functions by carrying functional ingredients into the coating matrix, such as antioxidants, antimicrobials, nutrients and flavors to further enhance food stability, quality, functionality and safety (*2–4*).

Berry fruits, such as strawberries and raspberries are highly perishable and have high physiological postharvest activities. As a consequence, they have short ripening and senescent periods that make marketing of fresh fruits a challenge. Their storage life is often terminated by fungal infection (*5*). The most prevalent method of maintaining quality and controlling decay is rapid cooling after harvest and storage at low temperatures for controlling moisture loss, postharvest respiration, and microbial growth (*5*). Edible coatings applied on the surface of berry fruits would provide an additional barrier for water and gas exchange with the environment. In addition, it can help delay decay incidence when using the coating material with anti-fungal property.

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Criteria for Developing Successful Coating

The success of applying an edible coating for extending shelf-life and enhancing quality of berry fruits depends on several factors (*4*), including

- Barrier properties of the coatings to moisture, oxygen, and carbon dioxide;
- Surface characteristics of the coatings, including coverage, adherence, glossiness, and coating wettability;
- Sensory properties of the coating materials, such as color, taste, etc.; and
- Antimicrobial and antioxidant properties of coating materials.

Since the major quality deteriorates involved in fresh berry fruits are via mass transfer phenomena, including moisture adsorption, oxygen invasion, flavor loss, and mold growth, permeation, absorption and diffusion to water, oxygen and carbon dioxide and prevention of mold growth are among the most important functional properties for edible coatings applied on berry fruits. These criteria in turn depend on the chemical composition and structure of the coating-forming polymers, the characteristics of the fruits, and the storage conditions.

Edible Coating Materials for Berry Fruits

Biopolymers, such as proteins, polysaccharides, lipids, and resins are the common coating forming materials that can be used alone or in combinations. The physical and chemical characteristics of the biopolymers greatly influence the functionality of resulting coatings. Selection of coating materials is generally based on their water solubility, hydrophilic and hydrophobic nature, easy formation of coatings, and sensory property. The properties of some common coating materials and their applications on fresh fruits and vegetables have been discussed in great details in the literatures (*4*, *6*), and are summarized below.

- Polysaccharides, such as cellulose, hydroxypropylcellulose (HPC), hydroxy propyl methyl cellulose (HPMC), starch, carrageenan, alginate, gum, chitosan. Overall, these materials have relatively high water permeability, and effective O_2 and CO_2 barriers at RH < 70 %.
- Proteins, including corn zein, soy protein, whey protein, collagen, gelatin, and casein. They are more permeable to water vapors, and have effective oxygen barriers at low RH.
- Lipids, such as beewaxes, fatty acids, and shellac. These materials have good water vapor barriers, but the coatings from these materials are usually too brittle and unstable when subjecting to different storage conditions, thus need to combine with polysaccharides or proteins for achieving desirable coating functionality.

The author's laboratory has examined several of the above coating materials for their applications on berry fruits with a special interest in chitosan. Chitosan, a linear polymer of 2-amino-2-deoxy-β-D-glucan, is a deacetylated form of chitin, a naturally occurring cationic biopolymer (*7*, *8*). It occurs as the shell component of crustaceans (crab and shrimp), as the skeletal substance of invertebrates, and as the cell wall constituent of fungi and insect (*9*). Chitosan has been one of the most promising coating materials for fresh produce because of its excellent film-forming property, broad antimicrobial activity, and compatibility with other substances, such as vitamins, minerals, and antimicrobial agents (*4*). Chitosan-based coatings have shown the effectiveness in delaying ripening and decreasing respiration rate of fruits and vegetables, and reducing weight loss, color wilting and fungal infection in bell pepper, cucumber, and tomatoes (*10–12*). Another very attractive function of chitosan is its broad anti-fungi property (*13–15*), by inducing a plant-defense enzyme, chitinase in plant tissues, which degrades fungal cell walls (*14*). In addition, chitosan-based coatings can carry high concentrations of vitamins and minerals for increasing the content of these nutrients in the fresh and frozen fruits without altering its anti-fungal and moisture barrier functionality (*2*, *3*).

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Examples of Edible Coating on Berry Fruits Studied in the Author's Lab

The following sections briefly reports the studies conducted in the author's laboratory by using edible coatings, especially chitosan based coatings for extending shelf-life, enhancing quality and nutraceutical benefit of berry fruits during post-harvest storage.

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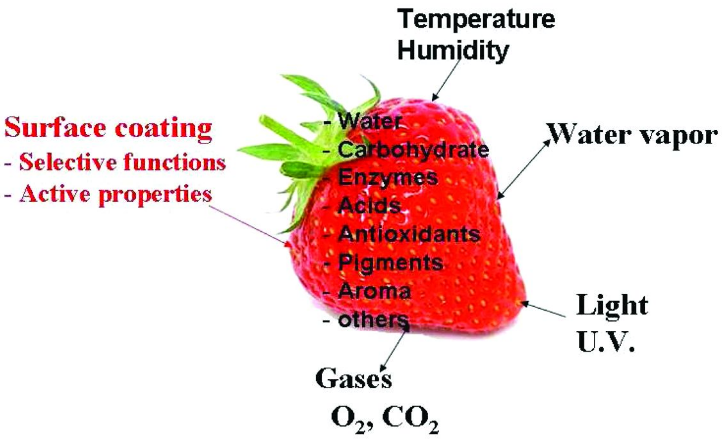
Temperature
Humidity
Surface coating
- Selective functions
- Active properties
- Water
- Carbohydrate
- Enzymes
- Acids
- Antioxidants
- Pigments
- Aroma
- others
Water vapor
Light
U.V.
Gases
O_2, CO_2

Figure 1. Edible coating with selective and active functions.

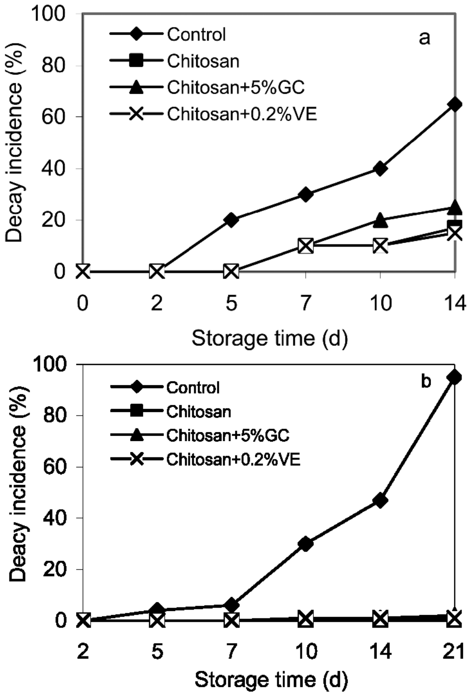

Figure 2. Effect of chitosan-based coatings on incidence of decay of fresh berry fruits stored at 2.0 °C and 88 % RH: (a) Strawberry (Puget Reliance); (c) Red raspberry (Tullmeen). GC = Gluconal® CAL, VE = dl-alpha-tocopheryl acetate. Reproduced with permission from reference (2). Copyright 2004.

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Table I. Calcium and vitamin E content in fruits coated with chitosan-based coatings containing calcium and vitamin E during fresh and frozen storage

	Calcium content x 10^{2} (g kg^{-1})		*Vitamin E content x 10^{2} (g kg^{-1})*	
Fresh	*Control*	*Coated*[+]	*Control*	*Coated*[++]
Strawberry (Puget Reliance)	23.63±1.62[a] (4.73%)	36.57±1.62[b] (7.31%)	0.34±0.09[a] (4.57%)	2.45±0.09[b] (32.69%)
Raspberry (Tullmeen)	22.03±2.92[a] (4.41%)	57.74±2.92[b] (11.55%)	1.15±0.13[a] (15.36%)	7.66±0.13[b] (102.13%)
Frozen	*Control*	*Coated*	*Control*	*Coated*
Strawberry (Totem)	21.24±0.85[a] (4.27%)	33.61±0.85[b] (6.72%)	0.40±0.05[a] (5.37%)	2.99±0.05[b] (39.85%)

[+] Chitosan coating containing 5% Gluconal® CAL [++] Chitosan coating containing 0.2% dl-α-tocopheryl acetate [a] Mean ± standand deviation of each treatment. Means with different superscript with each row indicate significant differences at $p<0.05$. [b] Mean ± standand deviation of each treatment. Means with different superscript with each row indicate significant differences at $p<0.05$. SOURCE: The table is derived from data presented in reference (*2*) by permission. Copyright 2004.

Table II. Consumer acceptability mean ratings1 of chitosan-coated strawberries (Diamante) after one-day storage at 2°C and 88~89% RH

Coating Treatments[+]	*FRESH*	*AA*	*LA*	*LAE*	*p-value*
Appearance liking	7.27[a] (1.29)	7.40[a] (1.33)	7.41[a] (1.28)	6.85[b] (1.46)	0.0003
Overall liking	6.08[a,b] (1.93)	6.36[a] (1.73)	6.08[a,b] (1.77)	5.70[b] (1.71)	0.0274
Flavor liking	5.55 (2.26)	6.07 (2.09)	5.74 (2.11)	5.47 (1.98)	0.1404
Sweetness liking	5.33 (2.29)	5.75 (2.13)	5.34 (2.01)	5.00 (1.95)	0.0544
Firmness liking	6.17 (1.90)	6.10 (2.07)	6.14 (1.75)	5.61 (1.96)	0.0780

[+] FRESH =Fresh, untreated fruit; AA=2% chitosan dissolved in 1% acetic acid solution; LA=2% chitosan dissolved in 1% lactic acid; LAE=2% chitosan dissolved in 1% lactic acid solution and mixed with 0.2% dl-α-tocopheryl acetate. [a] Mean ± standand deviation of each treatment. Means with different superscript with each row indicate significant differences at $p<0.05$. [b] Mean ± standand deviation of each treatment. Means with different superscript with each row indicate significant differences at $p<0.05$. SOURCE: The table is derived from data presented in reference (*3*) by permission. Copyright 2005.

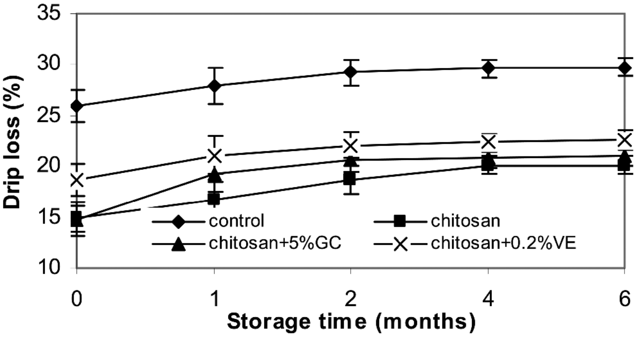

Figure 3. Effect of chitosan-based coatings on the drip loss of frozen-thawed strawberries (Totem) stored at –23 ºC up to 6 months. Vertical bars indicated standard deviation. GC=Gluconal® CAL, VE= dl-alpha-tocopheryl acetate. Reproduced with permission from reference (2). Copyright 2004.

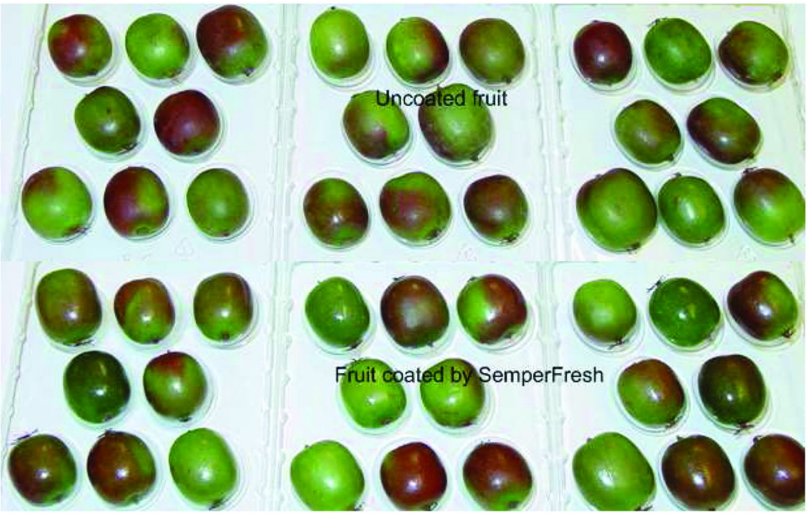

Figure 4. Hardy kiwifruit without (top row) and with Semperfresh™ coatings (bottom row) during refrigeration storage.

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Edible Coatings to Improve Storability and Enhance Nutritional Value of Fresh and Frozen Strawberries (*Fragaria ananassa*) and Raspberries (*Rubus ideaus*)

Chitosan-based edible coatings were used to extend the shelf-life and enhance the nutritional value of strawberries (*Fragaria* × *ananassa*) and red raspberries (*Rubus ideaus*) stored at either 2 °C or 88% relative humidity (RH) for 3 weeks or −23 °C up to 6 months. Three chitosan-based coatings (chitosan, chitosan containing 5% Gluconal® CAL, and chitosan containing 0.2% dl-α-tocopheryl acetate) were evaluated. It was found that adding high concentrations of calcium or Vitamin E into chitosan-based coatings did not alter their anti-fungal functions (Figure 2). The coatings significantly decreased decay incidence from over 60% to less than 20% for strawberries at the end of 2 weeks of cold storage, and from over 90% to less than 5% for red raspeberries at the end of 3 weeks of cold storage (Figure 3). Coating also reduced weight loss and delayed the change in color, pH and titratable acidity of strawberries and red raspberries during cold storage. Coatings also reduced drip loss (data not shown) and helped maintain textural quality of frozen strawberries after thawing. In addition, chitosan-based coatings containing calcium or Vitamin E significantly increased the content of these nutrients in both fresh and frozen fruits. One hundred grams of coated fruits contained about 34–59 mg of calcium, or 1.7–7.7 mg of Vitamin E depending on the type of fruit and the time of storage, while uncoated fruits contained only 19–21 mg of calcium or 0.25–1.15 mg of Vitamin E (Table I) (*2*).

Consumer sensory and trained sensory panel studies were also conducted. Chitosan coatings did not change consumer acceptability of flavor, sweetness, or firmness of the samples based on a 240 consumers sensory study (Table II) (*3*). Trained panel results after 1 week of refrigeration storage showed that chitosan coated strawberries have similar sensory descriptors as those of fresh berries, while coatings containing vitamin E developed the waxy-and-white surface of the samples. The trained panel did not detect astringency difference among all samples, indicating that 1% chitosan coating did not change astringency of strawberries.

Edible Coatings to Improve Fruit Quality and Storage Life of Hardy Kiwifruit (*Actinidia arguta 'Ananasnaya'*)

Hardy kiwifruit (*Actinidia arguta* (Siebold & Zucc.) Planch. ex Miq)) have smooth, edible skins, and belong to the family of berry crop. They are not picked vine ripe, as they would be too soft to package and ship (*16*). Instead they are picked when physiologically mature and firm, and are stored under refrigeration (0 °C, 90-95% RH). Commercially, both high-vent and low-vent clamshell containers are used for packaging the fruits with the low-vent containers preventing fruit dehydration during transportation and storage, but significantly high cost. Edible coatings were evaluated for their capability of extending shelf-life of post-harvest fruit by monitoring the changes of fruit physicochemical qualities under commercial storage conditions, and their impacts on the sensory quality of the fruit using a consumer sensory study (*17*). Calcium caseinate, chitosan, PrimaFresh® 50-V (a vegetable-oil based coating, free of mineral hydrocarbons), and Semperfresh™ (a mixture of sucrose esters of fatty acids, sodium carboxymethlcellulose, and mono-diglycerides of fatty acids) edible coatings were investigated in 3-consective seasons during 2004-2006. Semperfresh™-coated and uncoated fruit were also evaluated by a sensory consumer panel using a hedonic scale. Coatings provided an attractive sheen (Figure 4) to the fruit surface and did not impair post-harvest ripening of fruit (Figure 5). The storage life of hardy kiwifruit may be extended to as many as 14 weeks through the use of low-vent packaging container combined with edible coatings. The consumer sensory test indicated that both coated and uncoated fruit were well liked (Table III). It was concluded that when appropriate coating materials are chosen, edible coatings can significantly improve the surface appearance of the fruit. Since low vent packaging container is costly, edible coatings may be of particular interest to producers who wish to improve their product quality while continuing to use high-vent packaging containers.

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Edible Coatings for Developing Ready-to-Eat Fresh Blueberries

Studies of using edible coatings for developing pre-washed, ready-to-eat fresh highbush blueberries (*Vaccinium corymbosum*) were conducted. Edible coatings of Semperfresh™ (SF), acid-soluble chitosan (ACH), water-soluble chitosan (WCH), calcium caseinate (CC), and blend of ACH with sodium alginate (SA) were evaluated for their effects on the fruit quality of fresh highbush blueberries (Duke and Elliott) under commercial storage conditions (*18*). Fruit were first washed in 200 ppm chlorinated water before applying coatings, packaged in high-vent (v) or non-vent (nv) clamshell containers, and then stored at 2 °C for 1 week, followed by storage at room temperature (20±3 °C) for up to 15 days for quality evaluation. Chitosan coatings, including ACH, WCH, and WCH+SA coatings helped reduce the decay rate of 'Duke' and 'Elliott' throughout the storage period (Figure 6). SF coating decreased weight loss of 'Duke' after 6 days of storage (data no shown). CC coating significantly increased firmness of 'Elliott' after 6 days of storage (data not shown). Compared with high-vent container, non-vent container reduced weight loss and increased firmness of fruit; for fruit packed in high-vent containers, coated fruit retained high firmness than those uncoated ones (Figure 6). In general, washed and coated 'Duke' fruit had a higher antioxidant (AC) and total phenolic (TPC) content than non-washed, control fruit (data not shown). Washing and coating did not affect AC and TPC of 'Duke' and 'Elliott'. The results suggest that edible coatings have potential for retaining quality of pre-washed, ready-to-eat fresh blueberries under commercial storage conditions.

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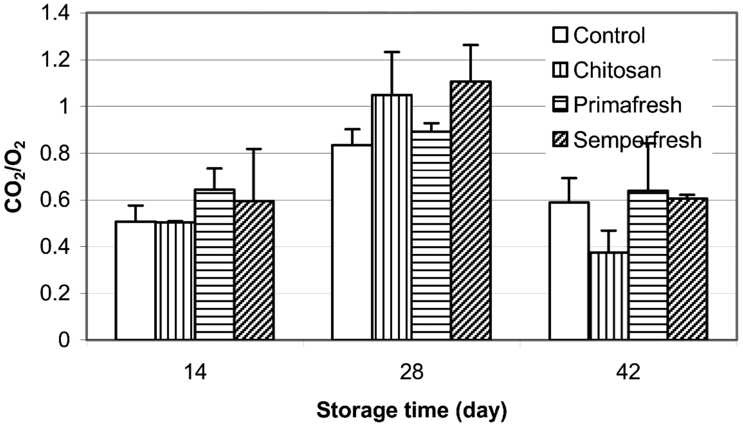

Figure 5. Headspace CO2/O2 ratio of 'Ananasnaya' hardy kiwifruit as affected by coating treatment during 6 weeks of storage at 2 °C in 2005 (mean ± SD). Coatings: Control = Uncoated, SF = Semperfresh™, ester based coating, PF = PrimaFresh® 50-V, vegetable-oil based coating, and CH = Chitosan based coating. Fruit were in LV packaging. Reproduced with permission from reference (17). Copyright 2008.

Table III. Sample "liking" means, standard deviations, and significance (n=91) of sensory consumer test for control and Semperfresh™ coated ripe 'Ananasnaya' hardy kiwifruit after storage at 2 °C for 3 weeks*

Sensory Attributes	*Control*	*SF-Coated*⁺	
	Sample Mean	*Sample Mean*	*p-value*
Appearance^{NS}	7.6 (0.9)	7.4 (1.1)	0.1866
Color^{NS}	7.4 (1.0)	7.4 (1.0)	0.9257
Overall Liking^{NS}	7.3 (1.1)	7.0 (1.7)	0.0823
Flavor^{NS}	7.1 (1.5)	7.0 (1.8)	0.3092
Sweetness^{NS}	7.2 (1.5)	7.0 (1.8)	0.2627
Sourness^{NS}	6.4 (1.8)	6.2 (1.9)	0.3245
Texture^{NS}	6.9 (1.7)	6.7 (1.9)	0.4278
Aftertaste^{NS}	6.0 (2.0)	5.6 (2.2)	0.1518

[*] Nine point liking (acceptance) scale where 1 = dislike extremely, 5 = neither like nor dislike, and 9 = like extremely. [+] Semperfresh™ coating. [NS] Attribute not significant at p>0.05 level. SOURCE: The table is derived from data presented in reference (*17*) by permission. Copyright 2008.

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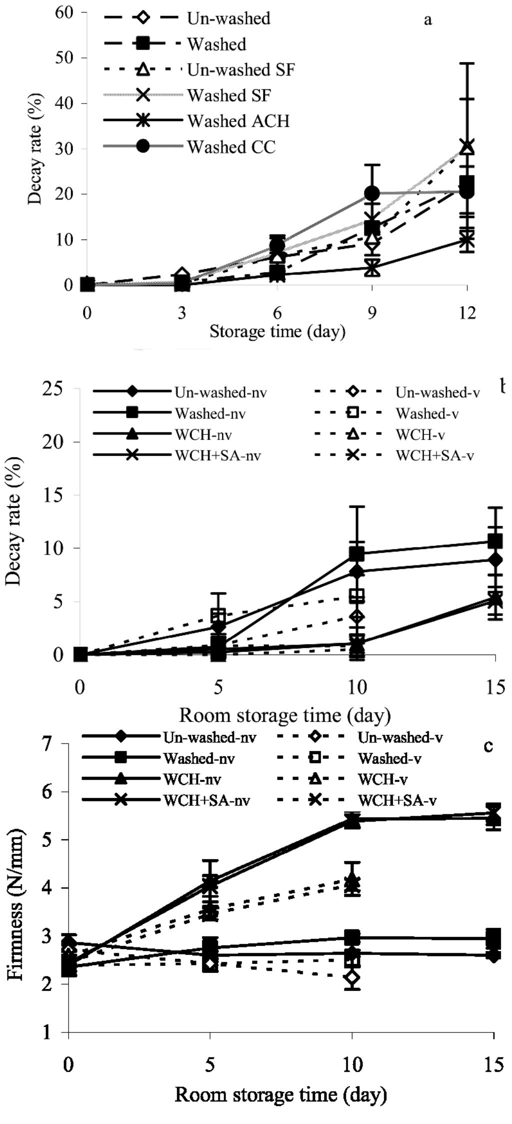

Figure 6. Decay rate of 'Duke' (a) and 'Elliott' (b) and firmness of 'Elliott' (c) blueberries during room temperature storage. Legend: -nv indicates non-vent container; -v indicates high vent container. Fruit qualities were not measured for the 'Elliott' packed in high vent containers on day 15 due to the severe shrinkage of the fruit.

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Summary

Results from the author's laboratory indicated that edible coatings, when applying appropriately, can effectively improve storability, enhance quality and nutraceutical benefit of fresh and processed berry fruits by retarding dehydration, improving texture quality, controlling microbial growth, and fortifying nutraceuticals. The key for success is using an appropriate coating material, packaging container, and method of applying the coatings. Different coating materials showed various effects on the post-harvest quality of fresh berry fruits. Chitosan coatings reduced rate of decay of strawberries, raspberries, and blueberries during refrigeration and room temperature storage. Semperfresh™ coating helped reduce weight loss and improve surface glossiness of fresh hardy kiwifruit and blueberries. Integration of different coating materials together, such as chitosan with sodium alginate can further improve the functionality of individual coating materials. High- and low-vent containers also showed their different impacts on the post-harvest storability of coated fruits. Depending on the type of coatings applied and expected fruit shelf-life, different packaing containers should be considered. The commonly used dipping method of applying coatings might reduce some benefits of coatings by removal of the natural waxy layer on the surface of some berry fruits; thereby new coating application methods such as spraying or brushing should be investigated.

References

1. Baker, R. A.; Baldwin, E. A.; Nisperos-Carriedo, M. O. *Edible Coatings and Films for Processed Foods*; Technomic Publishing Company, Inc.: Lancaster, PA, 1994; pp 89−104.
2. Han, C.; Zhao, Y.; Trabe, M. *Postharvest Biol. Technol.* **2004**, *33* (1), 67–78.
3. Han, C.; Lederer, C.; McDaneil, M.; Zhao, Y. *J. Food Sci.* **2005**, *70* (3), S172–178.
4. Lin, D.; Zhao, Y. *Compr. Rev. Food Sci. Food Saf.* **2007**, *6* (3), 60–75.
5. Ghaouth, A. E.; Arul, J.; Ponnampalam, R.; Boulet, M. Chitosan coating effect on storability and quality of fresh strawberries. *J. Food Sci.* **1991**, *56*, 1618–1620.
6. Zhao, Y.; McDaniel, M.*Sensory Quality of Foods Associated with Edible Films and Coating System and Shelf-Life Extension*; Elsevier Academic Press: U.K., 2005; pp 434−453.
7. BeMiller, J. N. *Chitosan*; Academic Press: New York, 1965; pp 103−106.
8. Davis, D. G.; Elson, C. M.; Hayes, E. R. *N,O-Carboxymethyl Chitosan: A New Water Soluble Chitin Derivative*. In 4th International Conference on Chitin and Chitosan, Trondheim, Norway, 1988.
9. Anon *Postharvest News Inform.* **1991**, *2*, 75.
10. El Ghaouth, A.; Arul, J.; Grenier, J.; Asselin, A. *Phytopathol.* **1992**, *82*, 398–402.

11. El Ghaouth, A.; Arul, J.; Ponnampalam, R. *J. Food Proc. Pres.* **1991**, *15*, 359–368.
12. El Ghaouth, A.; Ponnampalam, R; Castaigne, F.; Arul, J. *HortSci.* **1992**, *27*, 1016–1018.
13. Allan, C. R.; Hadwiger, L. A. *Exp. Mycol.* **1979**, *3*, 285–287.
14. Hirano, S.; Nagao, N. *Agri. Biol. Chem.* **1989**, *53*, 3065–3066.
15. Stossel, P.; Leuba, J.C. *Phytopathologische Zeitschrift (J. Phytopathol.)* **1984**, *111*, 82–90.
16. Strik, B.; Hummer, K. ‘Ananasnaya’ hardy kiwifruit. *J. Amer. Pom. Soc.* **2006**, *60*, 106–112.
17. Fisk, C. L.; Strik, B.; Zhao, Y. *Postharvest Biol. Technol.* **2008**, *47* (3), 338–345.
18. Wu, R.; Duan, J.; Lin, D.; Strik, B; Zhao, Y. *Postharvest Biol. Technol.* **2009**, submitted for publication.

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Chapter 19

Metabolic Engineering in *Fragaria x ananassa* for the Production of Epiafzelechin and Phenylpropenoids

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To confirm the *in vivo* function of a recently cloned strawberry UDP-glucose:anthocyanidin glucosyltransferase (*FaGT1*) gene, we downregulated its expression in strawberry fruit with a transient RNA interference (RNAi) method. In about one third of the injected fruits this led to a significant downregulation of *FaGT1* transcript levels consistent with reduced concentrations of anthocyanin pigments. In contrast, significant levels of epiafzelechin - formed by anthocyanidin reductase (ANR) from pelargonidin - were identified in *FaGT1* silenced fruits. Thus, the redirection of the metabolic flux towards the flavan-3-ol through downregulation of *FaGT1* offers a new method to increase the levels of this bioactive metabolite in fruit crops. In addition a dormant biosynthetic pathway of strawberry volatiles was uncovered by using the transient RNAi system. Silencing of the flavonoid pathway by downregulation of the chalcone synthase gene (*FaCHS*) provided phenylpropenoids for the biosynthesis of chavicol and eugenol in the fruits. These studies serve as foundation for metabolic engineering of strawberry flavor.

Strawberry (*Fragaria x ananassa*) is one of the most popular fruit crops worldwide and is grown in all temperate regions of the world. Much of the popularity of this fruit is due to the attractive flavor and the deep red color. In

In Flavor and Health Benefits of Small Fruits; Qian, M., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2010.

addition to traditional nutrients such as carbohydrates, vitamins and minerals, strawberries are also rich in phenolic compounds such as flavonoids e.g. epiafzelechin, which is the focus of intense study due to its proliferative effects on osteoblastic cells and selective inhibitory activities against cyclooxygenase-1 (COX-1) over COX-2 (*1*, *2*). The majority of flavonoids in strawberries are anthocyanins, the compounds responsible for the blue, red and purple hues of berries, grapes and other fruits.

The genetic and biochemical information about the last steps in anthocyanin biosynthesis in strawberry fruit is still limited although the chemical composition of the anthocyanins has been studied in detail (*3*). Stable products of the anthocyanin pathway are formed when a glycosyltransferase attaches a sugar molecule to the hydroxyl group at position 3 on the anthocyanidin aglycones. Anthocyanin concentration and flavonoid 3-*O*-glucosyltransferase activity increase in parallel during fruit ripening (*4*). Recently, anthocyanidin glucosyltransferase genes have been isolated from strawberry fruits and the recombinant proteins have been characterized (*3*, *5*).

Anthocyanidin 3-*O*-glucosyltransferases have also been isolated from grapes, maize and flowers of many ornamental plants in which anthocyanins are the major determinants of flower color (*6*). The genome of the model plant *Arabidopsis thaliana* contains over 100 putative glycosyltransferase sequences, and most of the respective proteins have been expressed heterologously. Out of 91 tested, 29 glycosyltransferases have been reported to accept the flavonol quercetin but only one enzyme from Arabidopsis glycosylates anthocyanidins (*7*).

Phenylpropanoid derivatives like eugenol are assumed to be key flavor components of wild strawberry. It is now known that acetates of hydroxycinnamyl alcohols are the precursors of phenylpropanoid-derived flavor compounds, and the corresponding eugenol synthase (*EGS*) and isoeugenol synthase (*IGS*) genes have been functionally characterized in both basil (*Ocimum basilicum*) and petunia (*Petunia hybrid* acv. Mitchell) (*8*, *9*). Since hydroxycinnamoyl derivatives accumulate after down-regulation of the chalcone synthase gene (*FaCHS*) in strawberry fruit, which is the branching point between the flavonoid and the phenylpropanoid pathway (*10*) we proposed that the anthocyanin pathway can be diverted for the production of phenylpropenes.

In the present study we down-regulated an anthocyanindin 3-*O*-glucosyltransferase gene in strawberry fruit with a recently developed RNAi method to confirm its function *in planta* and successfully redirected the flavonoid pathway to the phenylpropene pathway by the simultaneous silencing of *FaCHS* and overexpression of either *EGS* or *IGS*.

Materials and Methods

Chemicals

Solvents and reference compounds were obtained from Sigma, Aldrich, Fluka, Riedel de Haën (all Taufkirchen, Germany), Merck (Darmstadt, Germany)

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or Roth (Karlsruhe, Germany). Anthocyanidins were purchased from Polyphenols Laboratories (Sandnes, Norway).

Construction of pBI-*FaGT1i*

A blunt-end PCR product of the coding sequence of *FaGT1* was produced using a high-fidelity polymerase (Finnzymes, Espoo, Finland). The same primers were used to subclone the expression vector. The amplicon was digested with SpeI yielding a fragment of ca. 500 bp which was used for ligation into the binary vector pBI121 that contained XbaI/NheI and SpeI/SacI (Ecl136II) restriction sites separated by an intron from strawberry (*10*) The procedure yielded the intron-hairpin construct pBI-*FaGT1i*.

Vector Construction of pBI-*EGS* and pBI-*IGS*

The *O. basilicum EGS* (accession DQ372812) and *P. hybrida IGS* (accession DQ372813) coding region were cloned into the binary vector pBI121 replacing GUS (*10*).

Plant Transfection

Transient transfection of strawberry fruit with *A. tumefaciens* strain AGL0 suspensions containing pBI-*EGS*, pBI-*IGS*, pBI-*CHSi* or pBI-*FaGT1i* was performed according to a published procedure (*10*). Strawberry (*Fragaria x ananassa*) cv. Elsanta plants were grown under standard conditions at 25°C and a 16 h photoperiod. Control experiments were carried out by injecting strawberry fruits with *Agrobacterium tumefaciens* AGL0 cells carrying a pBI-Intron control construct (*10*).

Extraction of Volatiles

Two g freeze-dried strawberry fruit powder was homogenized with 20 mL of water and centrifuged (5000 g, 10 min). Phenol (10 µg) was added as internal standard. The supernatant was loaded onto an Amberlite XAD-2 polymeric adsorbent (20-60 mesh; 100 g; Aldrich) column. The column was rinsed with 50 mL water and volatiles were eluted with 70 mL diethyl ether. The extract was dried over anhydrous sodium sulphate, concentrated using a Vigreux column, reduced to 50 µl under a stream of N_2 and were analyzed by GC-MS.

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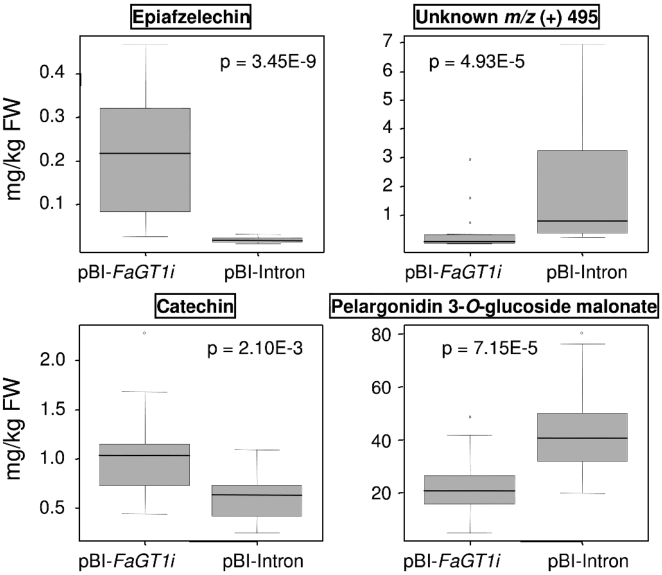

Figure 1. Metabolite levels in strawberry fruits injected with Agrobacterium tumefaciens harboring pBI-FaGT1i for the down-regulation of FaGT1 and in control fruits injected with pBI-Intron. FW fresh weight.

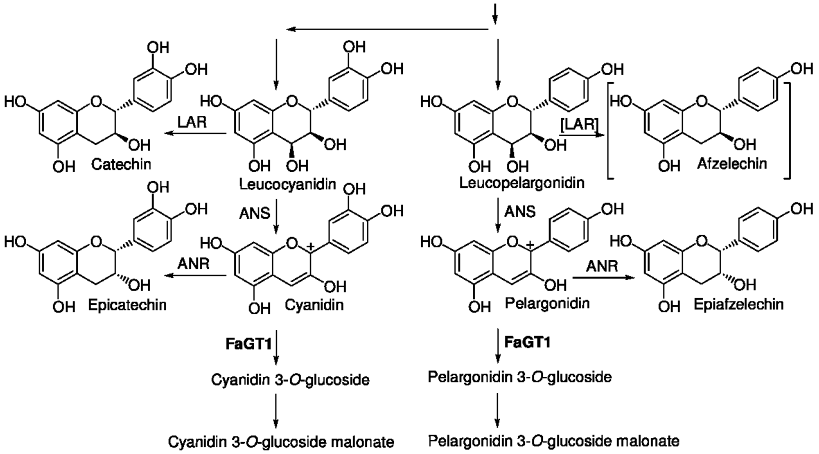
Catechin
LAR
Leucocyanidin
Leucopelargonidin
[LAR]
Afzelechin
ANS
ANS
Epicatechin
ANR
Cyanidin
Pelargonidin
ANR
Epiafzelechin
FaGT1
FaGT1
Cyanidin 3-*O*-glucoside
Pelargonidin 3-*O*-glucoside
Cyanidin 3-*O*-glucoside malonate
Pelargonidin 3-*O*-glucoside malonate

Figure 2. Section of the flavonoid/anthocyanin pathway illustrating the accumulation of epiafzelechin, epicatechin and catechin due to the reduced activity of FaGT1. LAR leucoanthocyanidin reductase, ANS anthocyanidin synthase, ANR anthocyanidin reductase, FaGT 1 Fragaria x ananassa glucosyltransferase

Metabolite Analysis

Freeze-dried strawberry powder (50 mg) was extracted with 250 µL methanol containing 0.2 mg/mL of the internal standars 4-methylumbelliferyl-β-D-glucuronide. Methanol was removed and the extract was re-dissolved in 35 µL water for analysis by LC-MS. Metabolite quantification was performed using the QuantAnalysis 1.5 software (Bruker Daltonics, Bremen, Germany) normalizing all results against the internal standard. Each analysis was performed in triplicate. Levels of metabolites determined in the RNAi experiment were displayed as box and whisker plots using the software SigmaPlot 8.0 (Systat Software, Erkrath, Germany). Statistical significance levels were calculated with the Wilcoxon-Mann-Whitney-U-Test (*11*) using the software package R (www.r-project.org).

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Results

Accumulation of Epiafzelechin

The full-length open reading frame of a UDP-glucose:anthocyanidin glucosyltransferase (*FaGT1*) was cloned from *Fragaria x ananassa* cv. Elsanta and heterologously expressed in *Escherichia coli* (*5*). The recombinant protein (FaGT1) glucosylated anthocyanidins (pelargonidin, cyanidin) and flavonols (kaempferol, quercetin) *in vitro*. To elucidate the substrates and function of the glucosyltransferase in strawberry fruit, the expression of *FaGT1* was down-regulated by RNA interference (RNAi) using a recently developed transient method (*10*). One third of the fruits injected with the RNAi-inducing vector pBI-*FaGT1i* were phenotypically different from fruits injected with the control construct pBI-Intron. The color of the RNAi fruits was generally less intense compared to the bright red color of the the control fruits. A significant down-regulation of *FaGT1* transcript expression was determined in the pBI-*FaGT1i* fruits in comparison to the transcript levels in control fruits.

Levels of anthocyanidins, flavonols and phenylpropanoid glucose esters were determined by LC-MS. To identify metabolites with significantly altered concentrations statistical methods such as the Wilcoxon-Mann-Whitney-U-test were applied. In fruits injected with pBI-*FaGT1i* the level of pelargonidin 3-*O*-glucoside malonate and pelargonidin 3-*O*-glucoside decreased significantly ($P<0.05$, Figure 1). We used the software packages MZmine and XCMS for differential analyses of the LC-MS data to explore also unexpected effects (*12*, *13*). Both computer programs revealed that the levels of epiafzelechin, catechin and an unknown compound (pseudomolecular ion *m/z* 495) were also significantly different in RNAi fruits when compared with control fruits (Figure 1).

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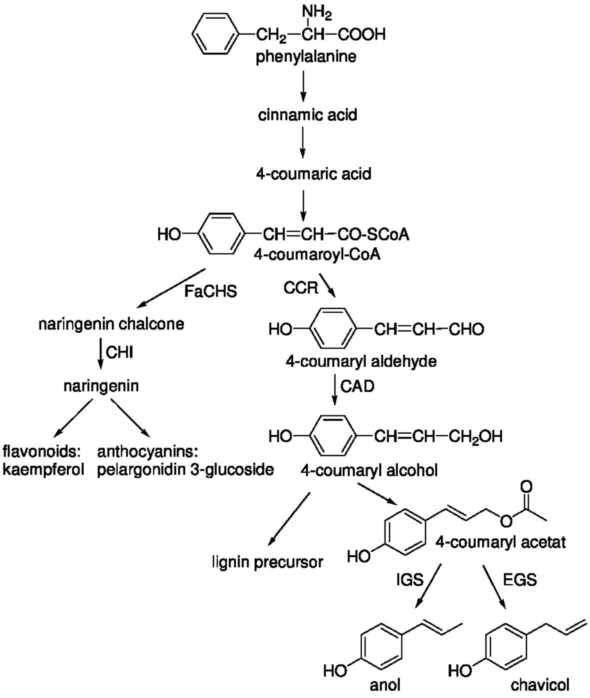
phenylalanine
cinnamic acid
4-coumaric acid
4-coumaroyl-CoA
FaCHS
CCR
naringenin chalcone
CHI
naringenin
4-coumaryl aldehyde
CAD
flavonoids: kaempferol
anthocyanins: pelargonidin 3-glucoside
4-coumaryl alcohol
lignin precursor
4-coumaryl acetat
IGS
EGS
anol
chavicol

Figure 3. Section of the flavonoid/anthocyanidin pathway demonstrating the formation of anol and chavicol. FaCHS F. x ananassa chalcone synthase; CHI chalcone isomerase, CCR 4-coumaroyl-CoA reductase; CAD 4-coumarylaldehyde dehydrogenase; IGS isoeugenol synthase; EGS eugenol synthase.

The results confirm that pelargonidin is a true substrate of FaGT1 *in planta* (Figure 1). Furthermore, down-regulation of *FaGT1* expression diverts the anthocyanin pathway towards the production of bioactive flavanols such as epiafzelechin which shows proliferative effects on osteoblastic cells and selective inhibitory activities against cyclooxygenase-1 (COX-1) over COX-2 (*1*, *2*). Recent data demonstrated that leucopelargonidin is not a substrate for leucoanthocyanidin reductase (LAR) which is in agreement with our observation that afzelechin was not formed after silencing of *FaGT1* (*3*).

Accumulation of Phenylpropenes

Recently, it has been shown that 4-coumaryl acetate accumulates when the chalcone synthase gene (*FaCHS*) is down-regulated (*10*, *14*). FaCHS is the branching point enzyme between the flavonoid/anthocyanin and the phenylpropanoid pathway (Figure 3). Besides, acetates of hydroxycinnamyl alcohols are the precursors of phenylpropanoid-derived flavor compounds such

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as anol, isoeugenol, chavicol and eugenol (Figure 3). The corresponding eugenol synthase (*EGS*) and isoeugenol synthase (*IGS*) genes have already been isolated and functionally characterized (*8*, *9*).

To redirected the carbon flux in cultivated strawberry fruit from anthocyanin pigment biosynthesis to the production of acetates of hydroxycinnamyl alcohols, which serve as the precursors of the phenylpropenes, we have down-regulated the level of *FaCHS* via RNAi-mediated gene silencing. Simultaneous heterologous overexpression of *EGS* or *IGS* genes in the same cultivated strawberry fruits in which *FaCHS* has been silenced enhanced the formation of eugenol, isoeugenol, and the related phenylpropenes chavicol and anol (Figure 4). The data show that *F. x ananassa* bears a phenylpropene biosynthetic pathway but the carbon flux is primarily directed to the formation of pigments. The level of eugenol and chavicol in cultivated strawberry can be enhanced through the overexpression of *EGS* and even more through the simultaneous downregulation of *CHS*.

The present paper reports the first metabolic engineering study in *F. x ananassa* and serves as a foundation for further investigations to optimize the production of valuable metabolites.

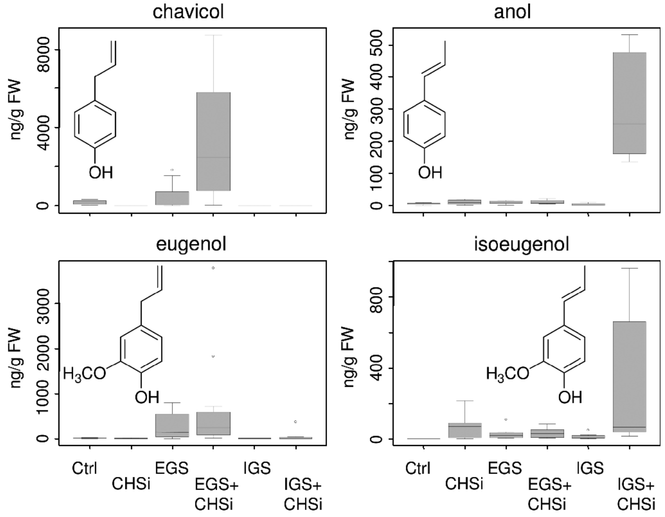

Figure 4. Metabolite levels in untreated control fruits (Ctrl) and in fruits in which CHS has been silenced (CHSi), or EGS (EGS) and IGS (IGS) have been overexpressed or in which CHS has been silenced and EGS (EGS + CHSi) or IGS (IGS + CHSi) have been simultaneously overexpressed.

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References

1. Su, B.-N.; Cuendet, M.; Hawthorne, M. E.; Kardono, L. B. S.; Riswan, S.; Fong, H. H. S.; Mehta, R. G.; Pezzuto, J. M; Kinghorn, A. D. *J. Nat. Prod.* **2002**, *65*, 163.
2. Chang, E. J.; Lee, W. J.; Cho, S. H.; Choi, S. W. *Arch. Pharm. Res.* **2003**, *26*, 620.
3. Almeida, J. R. M.; D'Amico, E.; Preuss, A.; Carbone, F.; de Vos, R. C. H.; Deiml, B.; Mourgues, F.; Perrotta, G.; Fischer, T. C.; Bovy, A. G.; Martens, S.; Rosati, C. *Arch. Biochem. Biophys.* **2007**, *465*, 61.
4. Given, N. K.; Venis, M. A.; Grierson, D. *J. Plant Physiol.* **1988**, *133*, 25.
5. Griesser, M.; Hoffmann, T.; Bellido, M. L.; Rosati, C.; Fink, B.; Kurtzer, R.; Aharoni, A.; Munoz-Blanco, J.; Schwab, W. *Plant Physiol.* **2008**, *146*, 1528.
6. Ford, C. M.; Boss, P. K.; Høj, P. B. *J. Biol. Chem.* **1998**, *273*, 9224.
7. Tohge, T.; Nishiyama, Y.; Hirai, M. Y.; Yano, M.; Nakajima, J.-I.; Awazuhara, M.; Inoue, E.; Takahashi, H.; Goodenowe, D. B.; Kitayama, M.; Noji, M.; Yamazaki, M.; Saito, K. *Plant J.* **2005**, *42*, 218.
8. Koeduka, T.; Fridman, E.; Gang, D. R.; Vassão, D. G.; Jackson, B. L.; Kish, C.M.; Orlova, I.; Spassova, S. M.; Lewis, N. G.; Noel, J.; Baiga, T. J.; Dudareva, N.; Pichersky, E. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 10128.
9. Vassão, D .G.; Gang, D. R.; Koeduka, T.; Jackson, B.; Pichersky, E.; Davin, L. B.; Lewis, N. G. *Org. Biomol. Chem.* **2006**, *4*, 2733.
10. Hoffmann, T.; Kalinowski, G.; Schwab, W. *Plant J.* **2006**, *48*, 818.
11. Hart, A *BMJ* **2001**, *323*, 391.
12. Katajamaa, M.; Miettinen, J.; Orešič, M. *Bioinformatics* **2006**, *22*, 634.
13. Smith, C. A.; Want, E. J.; Tong, G. C.; Abagyan, R.; Siuzdak, G. *Anal. Chem.* **2006**, *78*, 779.
14. Lunkenbein, S.; Coiner, H.; de Vos, R. C. H.; Schaart, J. G.; Boone, M. J.; Krens, F. A.; Schwab, W.; Salentijn, E. M. J. *J. Agric. Food Chem.* **2006**, *54*, 2145.

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